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RELATIONS BETWEEN METABOLISM AND MORPHOGENESIS DURING REGENERATION IN TUBIFEX TUBIFEX I

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There have been two prime viewpoints regarding the relation of metabolism to morphogenesis. One advanced by Child and his students has considered rate of metabolism in relation to the organismal factors initiating and directing the course of morphogenesis. Barth (1938, 1940a, 1940b), supporting Child's view, has suggested that increased oxygen tension in the tissues adjacent to a wound may furnish a primary stimulus for regeneration in hydroids; but he found that rate of regeneration was much more markedly stimulated by high oxygen tension than was rate of oxygen consumption. Thus the effect on regeneration may not have been mediated through an effect on total aerobic metabolism. The other viewpoint, advanced by Tangl (1909) and more recently by Tyler (1933, 1936, 1942), has considered rate of metabolism in relation to the release and utilization of energy in morphogenetic processes, particularly differentiation. Tyler concluded from his work on sea urchin embryos that differentiation requires the expenditure of metabolically released energy in addition to that required for maintenance, and that this energy does not become resident in structure but is released as heat during morphogenesis. This view is consistent with the work on "activity metabolism" (cf., Fisher et al., 1942, 1944).

The present study was undertaken to analyze further the relations between metabolism and morphogenesis from both viewpoints. In the regenerating annelid, measurements of morphogenesis may be made by counting the number of new segments produced (Stone, 1932 and Coldwater, 1933). The formation of a new segment is not a simple event, but involves distinct stages. This allows estimation of the rates at which various processes in regeneration proceed. Metabolism may be separated into fractions by means of poisons, and the relation of the activity of particular cellular respiratory systems to morphogenesis may then be tested. Two modes of approach were used in this study: oxygen consumption and respiratory sensitivity to poisons were measured at various times during regeneration (results reported in this paper), and the effects of continuous poisoning and of high and low oxygen tension were determined by measuring the progress of regeneration (results to be reported later).

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Materials and Methods

Worms of the species *Tubifex tubifex* [1] were rigorously selected for uniform size and condition (3.5 to 5.0 centimeters in length, a light, smoothly graded color, no signs of previous regeneration, no signs of breeding condition). For a week before use and between determinations the worms were kept in frequently changed tapwater at about 17°C. A 0.2 per cent solution of chloretone was used as an anaesthetic during amputation of the posterior two-fifths of each worm and during examination under the microscope. Individual worms were kept in anaesthesia no longer than ten minutes every third day (cf., Stone, 1932). Stage and rate of regeneration were estimated at intervals of several days. Three stages in the formation of a new segment were clearly distinguishable *in vivo*. First the new segment appeared as a narrow shadow perpendicular to the longitudinal axis of the worm. Later the new segment was slightly longer and the septum appeared as a pair of fine sharp lines. Finally the new setae were visible as small refractile bodies. The three stages were considered to be stages of localization, early differentiation, and later differentiation respectively (cf., Stone's description of the cytology and histology of regeneration in *Tubifex tubifex*). The number of segments in each of the stages was recorded separately. After regeneration had proceeded for a week or more, all stages of segments were present in the new tail, but segments which were hard to classify were few in number. "Rate of localization" [2] was calculated as the increase in total number of segments per worm per day; "rate of early differentiation" as the increase in number of segments in all stages of differentiation, and "rate of later differentiation" as the increase in number of segments with setae.

Most of the determinations of oxygen consumption were made by the Warburg manometric method, at 25°C. Worms for these determinations were taken in groups of twenty-five to forty, collected into a ball, rolled on filter paper to free them of excess water, placed in a pan of platinum foil and weighed quickly on a torsion balance. Consecutive weighings were found to deviate by less than one per cent. A difficulty in use of intact animals in the Warburg apparatus was encountered in the differences in degree of dispersion of the worms over the bottom of each flask. Probably the marked ability of *T. tubifex* to contract "oxygen debt" (Harnisch, 1935) was brought into play when the worms remained in a ball (average oxygen consumption in five determinations during which the worms remained in a ball was 0.12 milliliter per gram wet weight per hour compared to 0.16 milliliter for dispersed worms). Fortunately the worms spread out uniformly in most cases and only those determinations throughout which the worms were uniformly dispersed were included when calculating averages.

Successive determinations were made on the same worms at intervals of several days and the progress of regeneration was also measured. That this routine was not unduly injurious to the worms was indicated by a normal rate of regeneration and a casualty rate only slightly higher than that of worms upon which no manometric determinations were made; also the oxygen consumption of intact worms used as control remained constant within the limits of error throughout each series of

[1] Identification confirmed by Dr. R. G. Stone, University of Kansas City.

[2] This is the same as rate of regeneration as used by Stone (1932) and Coldwater (1933), although these authors did not calculate rate on a daily basis. From their published data on comparable worms the curve in Graph 1 may be duplicated.

determinations. These intact worms were anaesthetized and examined as were the experimental animals, injured individuals being removed from the group. About 10 per cent of the worms in each group had been removed by the end of the experiments.

A modification of the method reported by Howland and Bernstein (1931) was used for measuring oxygen consumption by individual worms. Each worm was drawn into a capillary tube 0.5 millimeters in diameter. By shifting the worm in a drop of water back and forth in the tube, air was drawn in at each end, then a drop of 5 per cent potassium hydroxide solution. Finally one end was sealed with paraffin oil and the other with vaseline (see Fig. 1). The tube was mounted in a

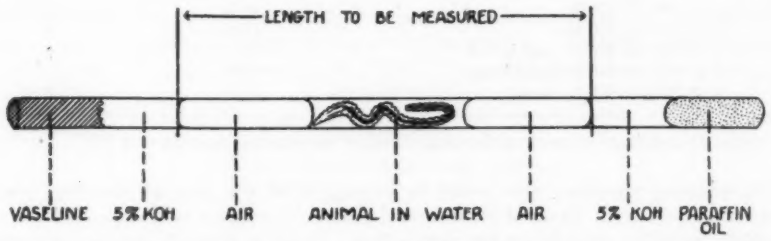


FIGURE. 1. Diagram of respirometer used in modified Howland-Bernstein method of measuring oxygen consumption (much enlarged).

vertical position on a glass plate in a glass-walled constant-temperature bath at 21°C. About fifteen such tubes were handled at a time. The distance between upper and lower air-KOH interfaces was measured using a horizontal microscope with a calibrated adjustment. The inside diameter of the capillary was measured exactly and the volume of oxygen consumed was calculated directly. A wormless tube served as a thermobarometer. The movement of the worm in the tube was found to furnish an efficient stirring mechanism in the fluid part of the system, and it was calculated that the capillary was not fine enough to hinder diffusion in the gaseous phase. After a series of readings, the capillary tubes were broken at the air spaces and the worms removed uninjured. Measurement of each worm was then carried out by anaesthetizing it and taking the diameter at four or five points and the length. The volume of the worm was computed as a series of cylinders.

DATA ON WORMS NOT UNDERGOING REGENERATION

As determined both by use of the Warburg apparatus and by the method just described, the rate of oxygen consumption of normal worms was within the range of rates reported by other workers using various methods (Dausend, 1931; Harnisch, 1935; Brazda and Rice, 1940). In agreement with the last named investigators, it was found that size of worms from 2.5 to 5.0 centimeters in length made no significant difference in rate of oxygen consumption. Concentrations of potassium cyanide from 2×10^{-3} M to 2×10^{-6} M had no inhibitory effect on oxygen consumption by normal worms (see Table I). Cyanide in a concentration of 2×10^{-2} M was rapidly lethal, but nevertheless did not decrease oxygen consump-

tion as drastically as its toxicity might suggest. Comparison with the data reviewed by Commoner (1940) shows that the total oxygen consumption by *T. tubifex* is of the same absolute magnitude as the cyanide-stable fraction of respiration in many other animals and tissues for which such data are available.

TABLE I

Oxygen consumption by intact worms in various concentrations of potassium cyanide

Solution	Q_{O_2}	Number of determinations
Tapwater	0.16	22
2×10^{-6} M KCN	0.16	2
2×10^{-5} M KCN	0.16	6
2×10^{-4} M KCN (non-lethal)	0.15	5
2×10^{-3} M KCN (semi-lethal)	0.19	2
2×10^{-2} M KCN (lethal)	0.11	1

Note: Determinations were made using standard manometric procedure at 25°C.

Respiratory quotient was found to average 0.68 for normal starving worms. The data agree with those of Brazda and Rice and suggest non-carbohydrate metabolism, possibly protein or fat metabolism. Such a type of metabolism is also expected from the cyanide-stable character of the respiration (cf., Commoner's review) as well as from the starving condition of the worms.

EXPERIMENTAL RESULTS

In preliminary work measuring the oxygen consumption of individual worms (see Table II) it was found that during the first few days of regeneration oxygen

TABLE II

Oxygen consumption by individual worms

Condition	Oxygen in mm.³ per mm.³ of worm per hour					
	0 days	1 day	8 days	11 days	15 days	19 days
Normal intact	0.10	0.10	0.11	0.09	0.09	0.09
Normal cut (regenerating)	0.10	0.12	0.14	0.17	0.15	0.15
X-rayed intact	0.08	0.09	0.07	0.09	0.08	0.11
X-rayed cut (inhibited)	0.10	0.11	0.09	0.11	0.10	0.11

Note: Determinations were made using capillary tubes at 21°C. Probable error was ±0.01.

consumption proceeds at a rate only slightly higher than normal. Later a marked increase in rate of oxygen consumption appeared: 0.08 mm.³ per mm.³ of worm per hour, or 85 per cent above normal. Subsequently the rate of oxygen consumption decreased. It was found that worms in which regeneration had been inhibited

by X-ray treatment showed no similar increase in oxygen consumption. It was considered that the increased oxygen consumption was associated with some process occurring during regeneration. In this preliminary work no counts of segments had been made, but the data of Stone (1932) were used for comparison. It was suggested that differentiation might be the process with which the increased oxygen consumption was associated. It was thought possible that the increase in oxygen consumption might be cyanide-sensitive as in the case of grasshopper embryos (Bodine et al., 1934, 1940). To test this possibility as well as to check results thoroughly using a standard method, experiments were set up in which the Q_{O_2} of regenerating worms in tapwater and in dilute cyanide solution (2×10^{-4} M KCN) was determined using Warburg manometers. Detailed observations of the progress of regeneration were made on the same worms. A total of nine groups of worms, 303 individuals, were followed through regeneration in three series of experiments. Four groups of worms, 137 individuals, served as control. Results of the manometric determinations are summarized in Table III, each figure being the average of

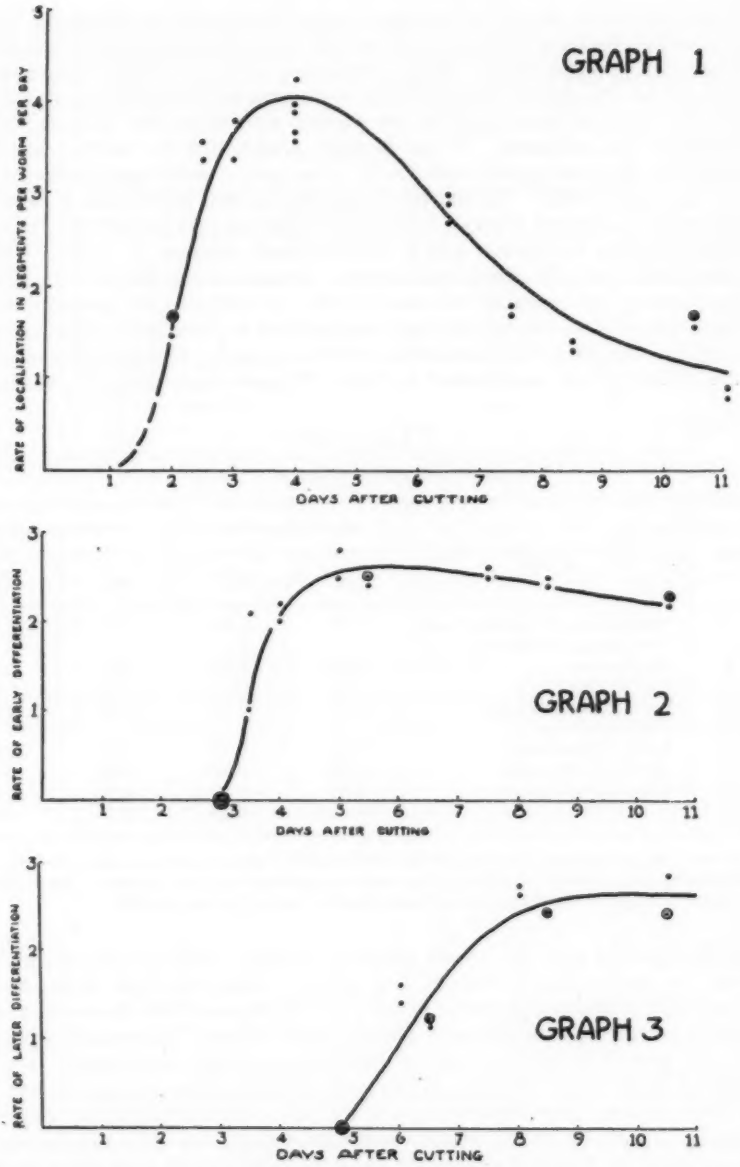
TABLE III

Oxygen consumption by groups of worms

Days	Predominating regenerative processes	Regenerating worms		Concurrent control	
		Q_{O_2}	$Q_{O_2}^{KCN}$	Q_{O_2}	$Q_{O_2}^{KCN}$
2	Mobilization of neoblasts and beginning localization	0.18	0.20	0.17	0.17
3	Localization	0.17	0.16	0.16	0.16
4	Localization	0.16	0.15	0.16	0.15
5	Localization and early differentiation	0.17	0.16	0.16	0.15
6	Early differentiation	0.16	0.15	0.17	0.17
8	Early differentiation	0.18	0.17	0.16	0.15
12	Later differentiation	0.24	0.24	0.16	0.15
13	Later differentiation	0.24	0.25	0.15	0.15

Note: The error when calculating from data on thirty-minute intervals was ±0.009. When calculating from data on the whole run, the error was reduced to about ±0.003. Mean deviation of separate averages from the grand averages listed in this table was ±0.008.

determinations on from two to five groups of worms. Data on progress of regeneration are summarized in Graphs 1, 2, and 3. After two days of regeneration, during which localization occurred in the first new segment from the neoblasts in the blastema, oxygen consumption was slightly above normal. Between three and five days, the period of maximal rate of localization, oxygen consumption was only slightly if at all above normal. While early differentiation was proceeding at its maximum rate six days after removal of the tail, oxygen consumption was strictly normal. But while later differentiation was proceeding, rate of oxygen consumption was increased: at twelve days the rate was 0.08 milliliters per gram per hour higher than the control, i.e., 50 per cent higher. At this time rate of later differentiation was at a maximum. The determinations using the Warburg method confirm in



Note: Mean deviation within each group of worms (twenty-five to forty individuals) ranged from 0.2 to 0.4 segments per worm per day, increasing with time.

general the results of the earlier determinations using individual worms in capillary tubes.[3] At no time was a significant cyanide-sensitive fraction of respiration found.

A summary of data on loss of weight by the worms involved in this study is given in Table IV. These data indicate not only that the regenerating worms lost

TABLE IV

Original length of worms in centimeters	Condition	Average weight per worm at first weighing after removal of tail (2, 3, 4 days)	Average weight per worm at last weighing (12, 13 days)	Average percentage loss per day
3.5 to 4.0	Regenerating	1.36 mg.	0.94 mg.	3.09
	Normal	1.92 mg.	1.57 mg.	1.82
4.0 to 5.0	Regenerating	2.07 mg.	1.57 mg.	3.01
	Normal	2.53 mg.	2.22 mg.	1.51

almost twice as much weight as the control, but also that the initial differences in weight between experimental and control groups due to removal of the tails cannot account for either the increased oxygen consumption or the increased loss of weight. The latter point is clear from a comparison between the larger regenerating worms (4.0 to 5.0 centimeters original length) and the smaller normal worms (3.5 to 4.0 centimeters in length).

DISCUSSION

Morphogenesis, the origin of form, involves the production of the specific structural chemicals of protoplasm, their orientation into submicroscopic structure, and the summation of submicroscopic structural differences into microscopic and macroscopic structure. Morphogenesis depends upon metabolism in that anabolic processes produce the particular compounds which become oriented into structural parts and catabolic processes must release in usable form such energy as may be required, if any, in morphogenetic processes. Morphogenesis may involve work in the thermodynamic sense according to either of two conditions: (1) there may be free energy of formation of the structure such that the energy becomes resident in chemical and physical structure, and/or (2) the synthesis of materials, their transport to particular positions, and their fixation in these positions, may require energy in such a way that the energy is ultimately dissipated as heat, even as the energy required to move an object horizontally from one point to another is ultimately dissipated as heat.

Tangl (1909), Farkas (1903) and Bohr and Hasselbalch (1903) defined "Entwichlungsarbeit," "work of development," as the total energy dissipated during embryonic development. In a number of different animals the Entwicklungsarbeit was found to be equal to about one-third of the total energy content of the unincubated egg. Needham (1931) criticized the concepts of Tangl and contended that

[3] The differences in general level of oxygen consumption are probably due to the difference in temperature at which determinations were made, i.e., Warburg determinations at 25° C., and capillary tube determinations at 21° C. (Brazda and Rice, 1940, found that oxygen consumption was increased about 40 per cent when the temperature was raised from 25° C. to 30° C.) and to inequality of unit volume to unit weight.

"work of development" should be a potential energy resident in structure, i.e., should be "work of differentiation." He further pointed out in reviewing the work of Bohr and Hasselbalch, that the energy resident in mechanical structure must be either non-existent or extremely small in amount. Essentially, the difficulties arose from the inadequacy of controls: no measure of an energetic cost of maintenance separate from an energetic cost of development was available in the work of Tangl, Farkas, and Bohr and Hasselbalch.

Tyler (1933) found that normally developing dwarf sea urchin embryos consumed more oxygen than normal-sized embryos in reaching the same stages of development. He interpreted his results in terms of a metabolic cost of differentiation. Tyler's concept, different from that discussed by Needham, involves the expenditure of energy in morphogenesis but not necessarily the storage of energy in visible structure. His investigations of giant embryos gave evidence consistent with his interpretation and he tried further to dissociate differentiation, growth, and maintenance in his material, but was unsuccessful. He stated (1936), "It has been concluded from earlier work that energy is required for the processes of embryonic differentiation, although the quantities involved could not be estimated."

Some of the difficulties encountered in studies of morphogenesis in embryonic development are avoided when morphogenesis is investigated in regeneration. Intact animals may be used in measuring the metabolism associated with maintenance,[4] and comparison between the metabolic rates of regenerating and of intact animals gives a measure of metabolism associated with morphogenesis. Increased rate of oxygen consumption during regeneration has been reported in Planarians (Coldwater, 1930). The additional metabolism appeared immediately after cutting and may have been associated with proliferation and increased numbers of the formative cells in this case. Coldwater chose this interpretation. However, it has not been demonstrated that either formative cells or other cells of an embryonic nature have intrinsically high respiratory rate. Tumor cells, for instance, have been found to have a relatively low Q_{O_2} (Warburg, 1930). Since these may be considered embryonic cells in which determination and differentiation are not proceeding, their metabolism may be considered entirely associated with maintenance and proliferation. On this basis, maintenance of embryonic cells should not, in itself, lead to increased respiration. From an examination of Needham's comprehensive review of the investigations of metabolism of embryos it is clear that the magnitude of rate of oxygen consumption is not greater than that of certain adult tissues. It is possible that in regenerating Planarians the additional metabolism was associated with truly morphogenetic processes rather than with proliferation and maintenance, but the data do not allow a distinction between the possibilities.

In the present work a distinction is possible. During regeneration in *T. tubifex* the rate of oxygen consumption was found to remain normal for the first week. During the second week the oxygen consumption was found to increase to well above normal. Because the markedly increased oxygen consumption did not appear during early stages of regeneration, it certainly was not associated with mobilization or proliferation of the neoblasts. That it was not associated with increase in size

[4] In the present work the data provide an empirical check on this assumption: since the Q_{O_2} remained normal during most of the first week of regeneration, the aerobic metabolic cost of maintenance of regenerating worms must be the same as that of intact worms.

of the new segments is clear from the consideration that increase in rate of oxygen consumption appeared before marked increase in the size of the new tail; during the first two weeks the regenerant tail constituted only a very small fraction of the total volume of the worm (less than one-twentieth). Hence the additional oxygen consumption may be considered to be associated with morphogenesis proper. From the present data it is not possible to distinguish whether the additional oxygen consumption occurred in the regenerant tail alone or whether the older tissues also consumed oxygen more rapidly. If the increase be referred to the regenerant tail alone, the Q_{O_2} of this part must have been extremely high. However, in estimating a metabolic cost of morphogenesis it is logical to refer the cost to the worm as a whole. Estimated on the basis of oxygen consumption, the metabolic cost of morphogenesis during the second week of regeneration would be at least half as great as the cost of maintenance of the whole worm. Another estimate of a possible cost of morphogenesis is available from the data. Because of the starving condition of the worms, all substrates of metabolism in both intact and regenerating worms were necessarily derived from the older tissues; loss in weight should be proportional to the amount of the materials metabolized away. Materials which were transferred from the older tissues to the regenerant tissues should not enter into the determination, and thus loss in weight should be proportional to net rather than gross breakdown of substrates. It was found that during the same periods of time regenerating worms lost weight almost twice as rapidly as intact worms. On this second basis, morphogenesis has a metabolic cost nearly the same as the cost of maintenance of the entire worm. The cost estimated from weight loss is thus greater than that estimated from oxygen consumption. Probably not all of the breakdown of substrates is associated with oxygen consumption, and morphogenesis may have an additional cost in terms of activity of anaerobic metabolism or glycolysis.

Analysis of a metabolic cost of morphogenesis may be carried further by comparing the time course of rate of oxygen consumption with the time course of events in regeneration, especially the time course of the processes which were measurable in terms of segments per worm per day. During the period of highest rate of localization, oxygen consumption was only slightly above that of non-regenerating worms. During the earliest stages of differentiation oxygen consumption was almost precisely the same as in non-regenerating worms. This indicates that localization is not particularly costly in terms of aerobic metabolism, while initiation of differentiation appears to cost nothing. The evidence does not indicate that localization and early differentiation are independent of catabolism. Measurements of total oxygen consumption give no indication of the relative activities of different fractions of catabolism, nor do they indicate how much energy may be released through cellular oxidative systems which use some hydrogen acceptor other than molecular oxygen. (Evidence which indicates an "activity metabolism" of localization will be presented in a later paper.)

The peak in rate of oxygen consumption was found to coincide in time with the peak in rate of later differentiation. Furthermore, the earliest time of marked increase in oxygen consumption coincided with the time at which the later stage of differentiation began appearing, first at a low rate, then more rapidly, parallel with increase in rate of oxygen consumption. The marked increase in oxygen consumption in regenerating *T. tubifex* is associated with differentiation, or some process

involved therein, and not with morphogenetic processes in general. It is suggested that differentiation is paid for in terms of metabolically released energy, and that the cost is high. As estimated from increase in oxygen consumption and in loss of weight, this cost must be at least half as great as the cost of maintenance of the entire worm.

SUMMARY

1. Methods of measuring "rate of localization," "rate of early differentiation," and "rate of later differentiation," during posterior regeneration in *Tubifex tubifex* have been described.

2. A method of measuring oxygen consumption of individual worms has been described.

3. Oxygen consumption, as determined according to the above mentioned method and also according to the Warburg manometric method, has been found to proceed at a near normal rate during localization and early stages of differentiation in the first week of regeneration.

4. Markedly increased rate of oxygen consumption has been found associated with maximum rate of later differentiation during the second week of regeneration.

5. No significant cyanide-sensitive fraction of respiration was found at any stage of regeneration.

6. Worms in which regeneration had been inhibited by X-ray treatment showed no increase in oxygen consumption.

7. Loss of weight by the starving regenerating worms was found to be almost twice as great as by the intact worms.

8. The data have been discussed in terms of a metabolic cost of differentiation, which cost would be at least half as great as the metabolic cost of maintenance of the entire worm.

9. It is concluded that the marked increase in aerobic metabolism observed during regeneration in *T. tubifex* is associated with some process or processes involved in differentiation.

The author expresses her deep appreciation for the direction and encouragement so generously given by Dr. Daniel Mazia, for the use of equipment belonging to the Department of Zoology, for the kindly interest of Dr. W. C. Curtis, and for the suggestions and criticisms offered by numerous people connected with the Department.

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THE EFFECT OF SEX AND AGE ON THE TEMPERATURE AT WHICH REVERSAL IN REACTION TO LIGHT IN ERISTALIS TENAX OCCURS

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Introduction

One of the most tantalizing phenomena facing the physiologist is the change of sign of reaction to light that occurs in many organisms. In this reversal an organism which is normally photopositive, moving toward the light, becomes negative and avoids the light, or a normally photonegative organism, moving away from the light, becomes positive. Some even consider this phenomenon "unapproachable," according to Maier and Schnierla (1935). Although this has claimed the attention of extremely able investigators for over 75 years, Holmes' statement (1916) holds good today. "The mechanisms involved are still unknown." The need for information concerning this problem is shown by the brevity of the paragraphs devoted to it by Wigglesworth (1939) and Heilbrunn (1943).

Reversal in reaction to light occurs "spontaneously" with no change in external conditions in Daphnia (Ewald, 1914; Clarke, 1932) and in Spondylomorum (Mast, 1918). It occurs rhythmically in some animals, according to Bohn (1905, 1907, 1909), Ewald (1914), and Warden, Jenkins, and Warner (1940), and in the course of normal development in others (Fraenkel and Gunn, 1940). For example, lobster larvae (Hadley, 1908) are positive for two days after hatching when they become negative and remain so until shortly before molting when they again become positive. They are negative in the early second stage and third stage but become positive before molting. In the fourth and later stages they are negative.

Various investigators maintain that a reversal of sign of the reaction to light can be produced in many organisms by the following changes: I. In the organism itself: metabolic rate, muscle tonus, water content, concentration of certain hormones, adaptation in sense organs, orientation to gravity, mode of locomotion (e.g., from swimming to crawling), mechanical stimulation, operations (such as removal of all or part of the wings or brain), training, and genetic constitution. II. In the immediate environment: temperature, food supply, background, light intensity (both gradual and rapid), wave length of light, osmotic pressure, oxygen pressure, hydrogen ion concentration, viscosity, and other changes produced by the addition of various inorganic and organic compounds.

Of all these methods none is more important than the use of alterations in temperature to produce changes in the sign of the reaction. An increase in temperature makes some photopositive organisms negative and some, that are negative, positive, while a decrease in temperature makes some positive organisms negative and some, that are negative, positive.

The animals and plants used in previous studies on the effect of temperature on reversal in reaction to light are as follows; swarm spores of *Haematococcus, Uloth-*

rix, and other algae (Strasburger, 1878); *Euglena, Volvox, Spondylomorum*, and other algae (Mast, 1911, 1918, 1927, 1932, 1936); the flagellate, *Chromulina* (Massart, 1891); *Rana clamata* (Torelle, 1903); *Arenicola* larvae (Kanda, 1919); *Polygordius* larvae (Loeb, 1893, 1905, 1906, 1918); *Lumbricus* and *Eisenia* (Prosser, 1934; Mast, 1936); *Cyclops, Cypris*, and a water spider (Mast, 1911); certain marine copepods (Loeb, 1893, 1905; Parker, 1901; Rose, 1929); *Daphnia* (Groom and Loeb, 1890; Loeb, 1906; Mast, 1911; Dice, 1914; Rose, 1929; Clarke, 1932); the copepod, *Leptodora* (Siedentop, 1930); *Artemia salina* (Bujor, 1911); *Balanus* nauplii (Groom and Loeb, 1890; Ewald, 1912; Rose, 1929); nauplii of the barnacle, *Chtamalus* (Rose, 1929); various amphipods (Phipps, 1915; Holmes, 1916); *Ranatra* (Holmes, 1905, 1916); *Notonecta* (Essenberg, 1915); the Mayfly nymphs, *Epeorus* and *Leptophlebia* (Allee and Stein, 1918); the beetle, *Anthrenus muscorum* (Janda, 1931); *Drosophila* (Carpenter, 1908); mosquito larvae, *Culex pipiens* (Miller, 1940); the tsetse fly, *Glossina morsitans*, and the stable fly, *Stomoxys calcitrans* (Jack and Williams, 1937).

The small number of species of insects tested furnishes no basis for Holmes' statement (1916), "In the insects reversal of the positive reaction is rather uncommon."

Because nothing is known about the effect of age and sex on the temperature at which reversal occurs in any organism, and because nothing whatever is known about reversal in *Eristalis tenax*, a study of reversal in reaction to light in this insect was made. For this work the drone fly proved as excellently adapted as it has for many other phases of physiological research.

In this paper are presented the results of a study of the effect of sex and age on the temperature at which reversal of reaction to light occurs. *Eristalis* at ordinary temperatures is highly photopositive. Preliminary experiments showed that between approximately 10°C. and 30°C. the flies crawl or fly directly toward a source of light. Outside these limits they are highly negative, moving directly away from a source of light. Since it was found impractical to study in detail the behavior of these flies at low temperatures but comparatively easy at high temperatures, this paper deals almost entirely with the latter.

Materials and Methods

The apparatus used (Fig. 1) consists of a box made of 6.3 mm. plywood (50 × 35 × 34 cm.). There are two main compartments, a light one, *A*, and a dark one, *B*. The light compartment is lined with white cardboard on the bottom, two sides and one end. The two compartments, 6.25 cm. deep, are separated by a wooden slide, *a*, painted white on the side toward the light compartment, which can be raised. Above the light compartment are two sliding glass panels, *b, c*, 6.3 cm. apart. A thermometer, *r* is inserted through a hole, *d* at the level of the white floor, *e*.

The dark compartment, *B*, has the same dimensions as the light compartment, but is lined with dull black cardboard and has a removable wooden cover, *f*, through the center of which a thermometer, *g*, is inserted. A glass window, *h* (3.8 × 12.5 cm.), covered by a removable shade on the outside, is at one end of this compartment.

Below the detachable floors, *i, j*, of each compartment the construction is identical. Five centimeters below these floors is a sheet of galvanized steel on which

rests a cardboard, *k*. Below the metal bottoms are two heat chambers, 12.5 cm. deep, *C*, *D*, lined with corrugated cardboard, *l*, and asbestos sheeting, *m*. The heat is supplied by two 100-watt Mazda lamps, *n*, with constant voltage, each lamp being wired separately.

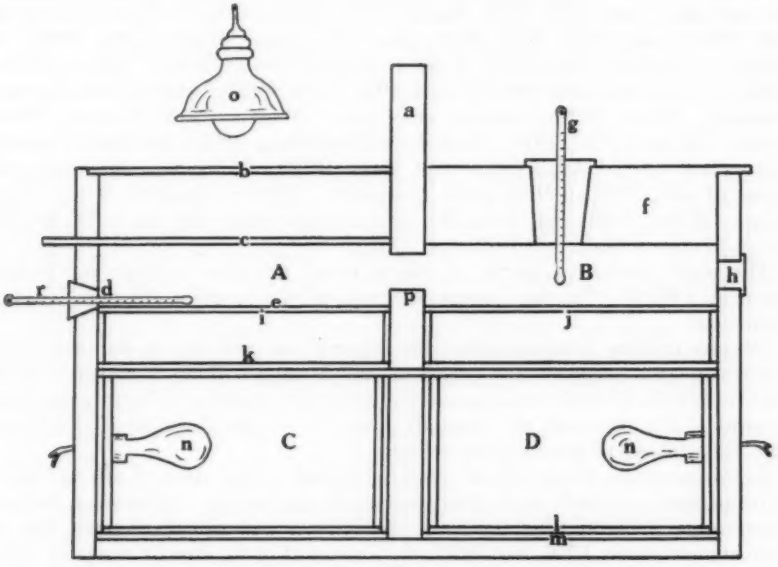

FIGURE. 1. Sectional view of the apparatus used. See text.

A 100-watt Mazda lamp, *o*, was suspended 81.25 cm. over the center of the white floor of the light compartment. The luminous intensity on this floor was 700 f.c., as recorded by a Weston exposure meter.

In these experiments only flies of known age and sex were used. They were raised in the laboratory according to the methods previously described (Dolley et al., 1937, p. 410). The exact temperature at which the flies became negative to light and went into the dark chamber was ascertained for six groups of flies whose sex and age are as follows:

1. Young males, 5–16 days.
2. Young females, 5–16 days.
3. Middle-aged males, 28–36 days.
4. Middle-aged females, 28–36 days.
5. Old males, 48–77 days.
6. Old females, 48–77 days.

Two hundred and seventy observations were made on members of each of these groups. No more than three were made on an individual insect and at least twenty-four hours elapsed between successive observations on the same fly.

All experiments were performed in a dark room. A typical experiment was made as follows: The temperature in the dark compartment was raised to between 35° C. and 37° C., that in the light compartment was raised to 28° C. Throughout the experiment the temperature in the dark compartment was seven to nine degrees higher than that in the light compartment. Ten to fifteen flies of the same age and sex, and with unclipped wings, were placed in the light compartment by sliding back the glass plates, *b, c*. These plates were replaced; the heating unit under the light chamber was turned on; and the center slide, *a*, was raised, making an opening (18 × 4 cm.) connecting the two chambers, *A, B*.

As the temperature in the light chamber rose the flies became restless, crawling and flying about. Soon individuals moved out of the light into the darkness of the dark chamber. A fly was considered to have reversed when it had passed completely beyond the center ridge, *p*. When this occurred the investigator recorded the temperature in the light compartment. The flies followed one another, and one by one entered the dark compartment. Frequently a fly returned to the light compartment after a few minutes in the dark, and then after a few seconds returned again to the dark compartment. Sometimes a given fly made three or four such successive reversals. The temperature at which the final reversal of a given fly took place was recorded and considered one observation. After approximately twenty-five minutes all the flies had reversed and entered the dark compartment. The organisms were then removed and those that had survived were placed in a cage with food and water. They were used for a maximum of two other experiments similar to that described above. An interval of at least twenty-four hours elapsed between successive tests on the same flies. About twenty-five per cent of the insects used in a given experiment did not survive their exposure to the high temperature of the dark compartment.

Results

The results obtained are given in Figure 2 and Table I. As is shown in this table, the mean temperatures in degrees centigrade at which the flies reversed are as follows: old males, 33.18 ± 0.09; old females, 33.822 ± 0.091; middle-aged males, 34.450 ± 0.088; middle-aged females, 35.06 ± 0.11; young males, 35.500 ± 0.099; young females, 36.106 ± 0.082. The standard deviations (Table I) are as follows: old males, 1.485 ± 0.064; old females, 1.489 ± 0.064; middle-aged males, 1.440 ± 0.062; middle-aged females, 1.742 ± 0.075; young males, 1.623 ± 0.070; young females, 1.318 ± 0.057.

Are the differences between the mean temperatures just given at which the two sexes reversed significant? According to Pearl (1940, p. 287), "the odds are 369.4 to 1 against the occurrence of a deviation in either the plus or minus direction as great or greater than 3 × S.E. These are long odds, and are conventionally regarded as amounting to practical certainty."

The differences between the means of the two sexes of the old, middle-aged and young flies are, respectively, 5 +, 4 +, and 4 + times the standard errors of the differences. This means that the odds against the occurrence from chance of these differences are, respectively, over 1,744,000; 15,770; and 15,770 to 1. It is evident that the differences between the means of the sexes in the above three age groups are clearly significant. Consequently, it is obvious that in each group the female flies reversed at a higher temperature than the male flies.

Age also has a definite effect on the temperature of reversal. This is shown when the observations on the individuals, both males and females, of the same age, are put together, as is done in Figure 2 and the lower portion of Table I. It is clear from this figure and table that the mean temperatures at which the old, middle-aged and young flies reversed are: 33.50 ± 0.07, 34.781 ± 0.071, and 35.786 ± 0.064, respectively. The differences between the means of the old and middle-aged, the old and young, and the middle-aged and young flies are, respectively, 12 +, 22 +, and 10 + times the standard errors of the differences. Consequently, age is an important factor in determining the temperature at which *Eristalis* changes its reaction to light. The younger the fly, the higher the temperature at which reversal occurs.

TABLE I

The effect of sex and age on the temperature at which reversal in reaction to light in Eristalis occurs. See text

Age	Sex	Mean temperature in degrees centigrade ± standard error	Standard deviation ± standard error
Old	male	33.18 ± 0.09	1.485 ± 0.064
	female	33.822 ± 0.091	1.489 ± 0.064
Middle-aged	male	34.450 ± 0.088	1.440 ± 0.062
	female	35.06 ± 0.11	1.742 ± 0.075
Young	male	35.500 ± 0.099	1.623 ± 0.070
	female	36.106 ± 0.082	1.318 ± 0.057
Old		33.50 ± 0.07	1.522 ± 0.046
Middle-aged		34.781 ± 0.071	1.652 ± 0.050
Young		35.786 ± 0.064	1.477 ± 0.045

It is patent that in the young flies the females with a standard deviation of 1.318 ± 0.057 showed less variation than did the males with a standard deviation of 1.623 ± 0.070; that in the middle-aged series the females showed greater variation than the males; that in the old flies the variation in the two sexes was about the same; and that between the three groups of different ages, each being composed of flies of both sexes, the young flies showed the least variation. The significance of the differences given above is not at present known, but it is true that the young flies were a more homogeneous group so far as age is concerned than were the old flies. The maximum difference in age between the members of the young series was eleven days, while the maximum difference in age between the members of the old series was twenty-eight days, a long period of time in comparison with the duration of life of *Eristalis*.

The reversals described above are unquestionably reversals to light and not to heat energy. The flies in going from the light compartment into the dark compart-

ment were not going from a region of high temperature to one of lower heat energy. The reading on the thermometer (Fig. 1, *r*) in the light compartment was compared with that of a black bulb thermometer made by coating a thermometer with lamp black. During the experiments the temperature as recorded by the latter was only 1.5 degrees higher than that recorded by the one used. Consequently, even if the flies had absorbed as much heat energy as did the black bulb thermometer, still the effective temperature in the dark compartment would have been from 5.5 to 7.5 degrees higher than that in the light compartment.

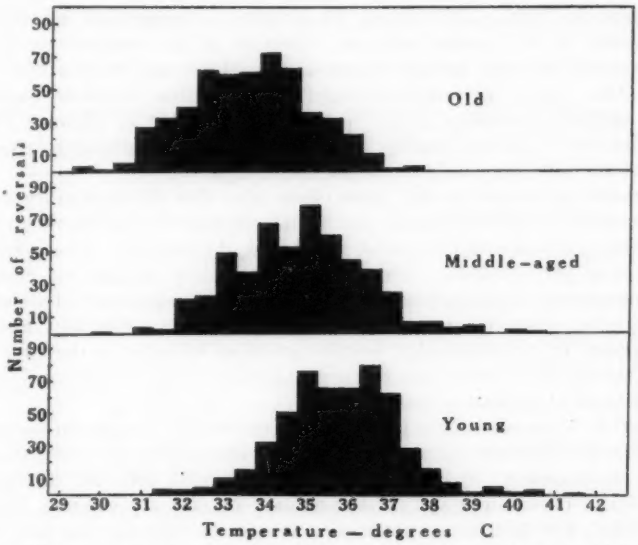

FIGURE. 2. Histogram showing the effect of age upon the temperature at which reversal in reaction to light in *Eristalis* occurs. Note that the means of the reversals for the older flies are at lower temperatures than are the means for the younger ones. See Table I.

DISCUSSION

The results presented in this paper show that the temperature at which *Eristalis* reverses in its reaction to light is not correlated specifically with either a decrease or an increase in heat energy for it can be caused by both. Reversal in sign of reaction occurs both when the temperature is raised above a certain point and when it is lowered below approximately 10°C., as stated previously.

This conclusion is in harmony with the following: 1. Mast's contentions (1918) that a decrease in heat energy and an increase in light energy produce similar effects in photopositive euglenae and other algae, and (1936) that reversal in *Volvox* and *Euglena* is due to "internal changes" and "is not specifically correlated with the immediate environment"; 2. the conclusion of Phipps (1915) that reversal in certain amphipods is associated with changes in their "physiological states"; 3. that of Welsh (1930) that reversal in the water mite, *Unionicola,* "is probably a central nervous phenomenon"; 4. that of Washburn (1936, p. 208) that the "complex influ-

ence of external and internal conditions on phototropism" . . . "is based on innate factors"; 5. that of Allee and Stein (1918) that reversal in reaction to light in certain Mayfly nymphs is associated with either increase or decrease in metabolic rate; and 6. those of Jack and Williams (1937) that if the temperature is raised sufficiently high the normally photopositive reactions of the tsetse fly, *Glossina morsitans*, and the stable fly, *Stomoxys calcitrans*, become reversed, and that "the temperature at which the negative reaction develops appears to depend somewhat upon the flies' physiological conditions."

The conclusion stated above is not in harmony with the following: 1. Davenport's contention (1908, p. 200) that a "Diminution of temperature below the normal causes reversal of the normal response, elevation of the temperature to near the maximum accelerates the normal response"; 2. Maier and Schnierla's conclusion (1935) that the sign of the reaction to light in an organism "depends upon the characteristic metabolic condition of its species"; and 3. that of Holmes (1905) for *Ranatra* that any condition causing an increase in activity accentuates positive reactions and any quieting conditions make them negative.

The results presented in this paper show also that the temperature at which reversal occurs in *Eristalis* depends upon the resistance of the organism to the injurious effects of temperatures above and below the normal. The nature of this resistance is as yet unknown. Old flies are less able to endure the effects of abnormal temperatures than are young ones. Since the temperature at which reversal occurs in a given intensity of light is lower in old than in young flies, and in males than in females, it is probable that females are more resistant to the effects of temperatures outside the normal range than are males. This conclusion is confirmed by the results of work now in progress.

Reversal in response to light in *Eristalis* produced by changes in temperature is probably due to a different mechanism from that involved in the reversals occurring in normal development, which are doubtless associated with the development or degeneration of photosensory or other organs. As stated previously (Dolley and Haines, 1930), *Eristalis* larvae are highly positive to light for the first few hours after hatching. They then become negative to light and remain so until they pupate. The imagos are at first negative but soon become positive at ordinary temperatures, and remain so throughout their lives. Similar phenomena have been described for blowflies (Herms, 1911; Gross, 1913; Patten, 1916), *Amaroucium* larvae (Grave, 1920; Mast, 1921), and for other organisms. According to Pause (1918) Chironomus larvae are positive until they have formed haemoglobin when they become negative.

Summary

1. Over 1,620 observations were made on over 1,000 flies in ascertaining the temperature at which *Eristalis tenax* becomes negative to light.

2. In a luminous intensity of 700-foot candles *Eristalis* is highly photopositive within a temperature range between approximately 10° and 30°C. Outside these limits it is highly negative. Reversal of the photopositive reaction can be produced either by increase or decrease of the temperature.

3. In high temperatures the temperature at which *Eristalis* changes in its reaction to light depends on the sex of the flies. Females cease their positive reaction to light and become negative at a higher temperature than do males.

4. In high temperatures the temperature at which *Eristalis* changes in its reaction to light depends also on the age of the flies. The younger the fly, the higher the temperature at which it ceases its positive reaction to light and becomes negative.

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OBSERVATIONS ON THE REPRODUCTION OF THE SPINY DOGFISH, SQUALUS ACANTHIAS *

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The elasmobranch fishes offer an exceptional opportunity for studies of the physiology of reproduction. Within this old and diversified group of vertebrates many species are oviparous, others are ovoviviparous, and some are viviparous, thus indicating various degrees of physiological dependence of the young on the parent. Ranzi and his collaborators have made comparative studies of several representative species and their results and the observations of other workers have been presented in several reviews (see Needham, 1942, for discussion and references).

Our observations were made on two species of dogfish, *Squalus acanthias* (spiny dogfish) and *Mustelus canis* (smooth dogfish), common in the vicinity of Woods Hole, Massachusetts. The spiny dogfish is ovoviviparous and maintains its young free in the uterus while the smooth dogfish is viviparous and has young whose yolk sacs are modified into placental structures that are firmly attached to the uterine endometrium during the last two-thirds of pregnancy. Our chief interest was a study of the endocrines that might be associated with gestation in these two species of dogfish. This approach was quite different from the investigations of Ranzi and others which were primarily concerned with foetal nutrition. However, it was found that *Squalus acanthias* was not suitable for our experimental purposes. They could not withstand necessary surgical procedures nor could they live for long periods though kept in large, outdoor live-cars. Consequently, the results of our studies on *Squalus acanthias* were mostly observational. These are reported here while the results of our experiments on *Mustelus canis* will be published separately.

General Observations on the Reproductive Cycle

The different times at which *S. acanthias* is present at various points along the Atlantic coast indicate that it migrates northward in the spring and southward in the fall. It appears in Buzzards Bay and Vineyard Sound in late April or early May. It becomes very abundant during May but declines in number by the end of the month and is caught only infrequently toward the close of June. Correlated with its disappearance from the vicinity of Woods Hole is its arrival at successive points farther north, reaching the coastal waters of Newfoundland the last of June or early July. The migration southward apparently begins in September and spiny dogfish are again caught in large quantities by fishermen at Woods Hole in October and November as they pass by Cape Cod.

Our studies are based on animals obtained during the spring and fall migration, a period of about six months. Consequently we can only infer what changes might

* This work was assisted by a grant from the Permanent Science Fund of The American Academy of Arts and Sciences.

have occurred in the reproductive system during the winter and very early spring. However, we have examined several hundred spiny dogfish since our investigations were started in 1936 and believe that we have a fairly accurate account of the reproductive cycle and duration of pregnancy. Some of our observations are corroborations of certain details of the reproductive cycle well known to fishermen and collectors at Woods Hole, while others confirm the results of similar studies on *Squalus acanthias* by Hickling (1930) and Templeman (1944) in Newfoundland, and by Ford (1921), Ranzi (1934) and Popovici (1938) on *Acanthias vulgaris* as found in the English Channel, Mediterranian Sea, and Black Sea.

Most of this work was done at the Oceanographic Institution in Woods Hole and we wish to express our sincere thanks for generous laboratory accommodations. We also are indebted to Dr. H. B. Bigelow and Dr. W. C. Schroeder for many helpful suggestions and to Mr. James McInnis of the Marine Biological Laboratory, Supply Department, who made it possible for us to examine many hundreds of dogfish.

Uterine Young

The sexually mature females, when they arrive at Woods Hole during May, can be divided into two distinct groups depending on the stage of development of their embryos. For convenience we can designate these two periods of gestation as Stage *A* and Stage *C* (Fig. 1). Those females belonging to Stage *A* have ovulated recently while those of Stage *C* have foetuses 12 to 20 cm. in length. Females that are 80 cm. to a meter or more in length are pregnant almost without exception and all are invariably in either Stage *A* or Stage *C* of gestation. The pregnant females are not always equally divided between these two stages. In 1937 they were about equal but in 1938 there were about four in Stage *A* to one in Stage *C*.

The uterine ova of animals in Stage *A* are enclosed in a membranous envelope forming a structure commonly called a "candle." There is usually a candle in both the right and left horn of the uterus and each contains one to four ova. The average wet weight of 26 candles containing one to 4 ova (total of 65 ova) was 46.2 grams per ovum. The development of the embryos is about the same, each ovum having a blastoderm somewhat comparable to that of a hen's ovum at about 16 to 20 hours incubation.

The pups of Stage *C* have large pendent yolk sacs and are free in the uterine lumen. The range of variation in length is considerable (12 cm. to 20 cm.) and also there is a great difference in the size of the yolk sac, even among pups of the same length. Consequently there is a wide variation in the combined weights of the pups plus their yolk sacs ranging between 30 to 60 grams wet weight.

When the dogfish return to the vicinity of Cape Cod in October and November, the adult females again can be divided into two groups depending upon the condition of pregnancy. These two groups have been designated as Stage *B* and Stage *D* (Fig. 1). The ova of Stage *B*, though much further developed than those of Stage *A*, are yet enclosed in the candle membrane. The embryos are from 3.5 to 7.5 cm. long and are connected by an umbilical stalk about one inch in length to a large yolk sac about the size of the original ovum. The average wet weight of the embryo plus the yolk sac is about 40 grams.

Some of the females of the other group, Stage *D*, have given birth and consequently their uteri are empty while those that have not given birth have large pups

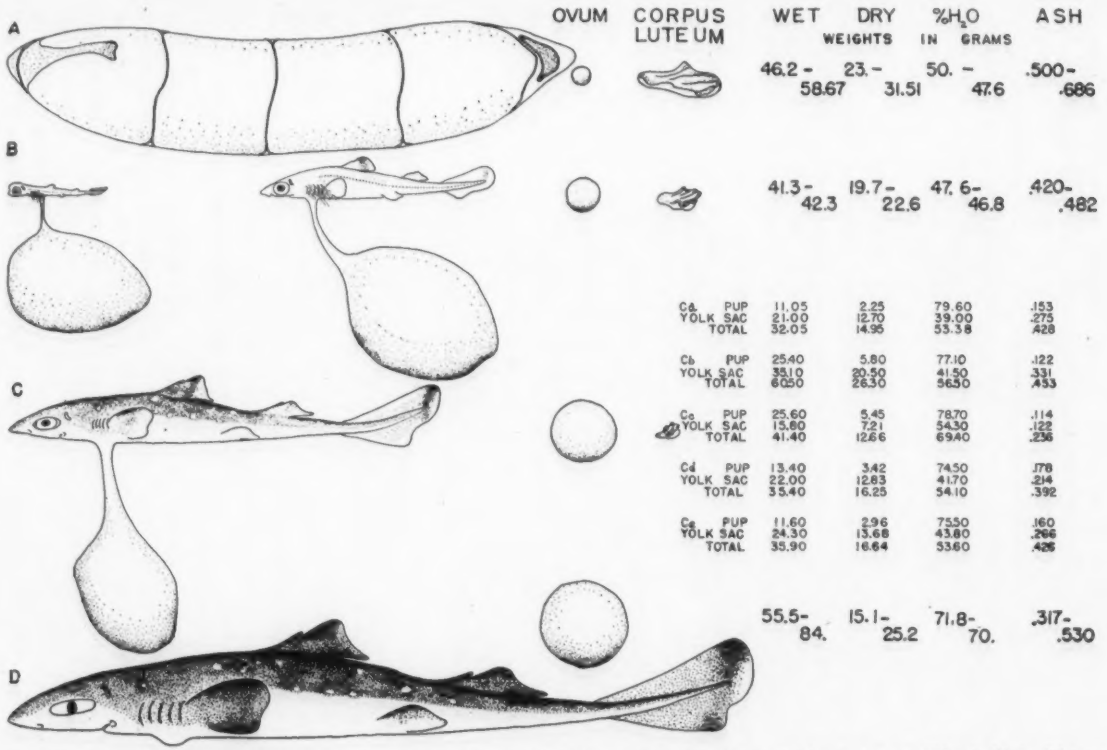

FIGURE 1. Comparison of the four stages of gestation in the spiny dogfish, *Squalus acanthias* as seen at Woods Hole, Massachusetts, in late April and May (Stages *A* and *C*), and October and November (Stages *B* and *D*). *Ovum.* Progressive increase in size of ovarian ova with the advancement of pregnancy. *Corpus luteum.* Gross appearance of the involuting corpora lutea. Drawings ¼ natural size.

that are 23 to 29 cm. in length. The yolk sac of some of these pups has been withdrawn into the body and the umbilicus is yet open while in others it is yet present though greatly reduced. The wet weight of such pups varies between 55 and 85 grams.

These four groups of uterine young obviously represent four different stages of gestation. When arranged in order of degree of development, which also agrees with the sequence of time, it readily is seen that the period of gestation is surprisingly long.

Ovarian Ova

When the ovaries of pregnant spiny dogfish representing the four stages of gestation are compared, they too show four corresponding stages in the development of the ovarian ova (Fig. 1). The early development of the embryos of animals of Stage *A* indicates that ovulation has occurred quite recently and consequently large ovarian ova are absent. Instead, there are numerous, usually 50 or more, small marble-white follicles the size of peas that contain ova usually weighing less than one gram. The embryos of Stage *B* are more advanced and likewise the ovarian ova. The largest eggs in the ovary are about the size of Concord grapes and have begun to take on a yellowish color due to the storage of yellow yolk.

The ovaries of Stage *C* females contain developing ova each of which usually weighs about 9 grams wet weight (variation 4 to 15 grams, average for 264 eggs, 9.1 grams). There are usually two or three such ova in each ovary, the total number never exceeding eight or nine, and they are not necessarily divided equally between the right and left side. They represent the next generation of ova that will be ovulated after the termination of the existing pregnancy and their average number is in close agreement with the average number of young found at one time in the uterus.

The average number of large ovarian ova for Stage *D* is about the same as that for Stage *C* but the eggs are considerably larger, being about 3 cm. in diameter and weighing between 30 and 40 grams. These weights are from two-thirds to three-fourths that of the candle ova of Stage *A* and strongly suggest that the eggs of the spiny dogfish at the time of ovulation probably average close to 50 grams. The fish of Stage *D* are seen at Woods Hole in October and November and the difference between the weight of the ovarian ova at that time and the ova in the candles of Stage *A* should give a fairly accurate estimation of the weight of the egg at ovulation, which probably occurs in February or March.

Thus it is seen that the ovaries of the four different groups show a progressive development of ovarian ova. Those of Stage *A* are small and numerous while in the succeeding three stages of gestation there is an increase in size and a decrease in number. It is, of course, quite common among the vertebrates for many small Graafian follicles to start developing even though only a very few ova are ovulated. In the dogfish as elsewhere the decrease in number of follicles is brought about by atresia. Most of such elimination must occur before Stage *C*, as we did not see large follicles that were undergoing atresia in either Stage *C* or *D*. Nor did we find large follicles in Stage *A* that had failed to ovulate and consequently were being resorbed.

Corpora Lutea

One of the interesting features associated with gestation in the dogfish is the formation of corpora lutea in the ruptured follicles following ovulation. Aside from the question of their physiological importance, the corpora lutea are useful in determining the time sequence of pregnancy as they undergo definite morphological changes as gestation proceeds.

The number of corpora lutea in the right and left ovary varies considerably. There may be six corpora in one ovary and none in the other or their distribution may be 4 and 4, 2 and 6, 3 and 3, 1 and 4, 2 and 4, etc. There is almost always an exact correlation between the number of corpora lutea and the number of embryos in the uterus. However, there is no correlation whatever between the number of corpora in any ovary and the number of embryos in the corresponding uterine horn on that side. The number of embryos in the candles of the right and left horns of the uterus tend to be evenly divided regardless of the unequal distribution of the corpora lutea between the ovaries. It seems that at ovulation an ovum may enter either horn of the uterus in a way probably similar to that described by Metten (1939) for *Scylliorhynus canicula.* However, in *S. acanthias* Templeman (1944) found, from a statistical study of several hundred animals that the number of young tends to be greater in the right uterus than in the left.

The corpora lutea of Stage *A* (Figs. 1, 2, 3) are large wrinkled sac-like structures 2 cm. or more across at the greatest diameter. Each is surrounded by a broad tissue space containing a clear fluid and consequently can be separated easily from the ovarian stroma and removed. Histological examination of such corpora shows a remarkable modification of the follicular wall which is thrown up in tall folds and drawn out in long lacy extensions into the follicular cavity. The luteal tissue seems to be derived entirely from the follicular granulosa.

The cells of the young corpus luteum first become very tall and have large vacuoles and irregular external cell borders (Fig. 2). They apparently have the ability to take up solid particles from the cavity of the ruptured follicle as occasionally a yolk granule is found in such cells. The ingestion of yolk by luteal cells is a common observation in the smooth dogfish and especially is this so during atresia of large follicles. Such activity of the corpus luteum will be described more fully in a subsequent report on the smooth dogfish.

The cells of the theca interna that are taken into the folds of the developing corpus do not take on a glandular appearance but contribute to the thin connective tissue framework that supports a rather generous supply of blood vessels. Cells derived from the granulosa are sharply separated from the underlying connective tissue structures.

There is considerable variation in the appearance of the granulosa-luteal cells of the corpora lutea of Stage *A* which seems to be correlated with age. The first reactions of the granulosa following rupture of the follicle and ovulation result in the development of the structure just described (Fig. 2). Other corpora are found, in animals of this stage, that seem to be older (Fig. 3). These are somewhat smaller and their walls thicker. The luteal cells have lost considerable vacuolation and are more tightly pressed into a folded epithelial-like lining of the luteal cavity.

Females with candle embryos taken in October and November, Stage *B*, have corpora lutea that are definitely older than those found in Stage *A*. This difference

in age is shown by a decrease in size of the luteal body and a reduction in size of the luteal cells which now have irregular shaped nuclei (Fig. 4). Also, those cells that lie next to the luteal cavity have become loosened and scattered through the luminal fluid, while the connective tissue of the folds has been modified into very conspicuous membranous partitions.

The corpora lutea of Stage *C* are smaller than those of Stage *B* and the luteal tissue is undergoing degeneration as shown by vacuolation and disintegration of the cells (Fig. 5). These changes must progress very slowly as the luteal bodies

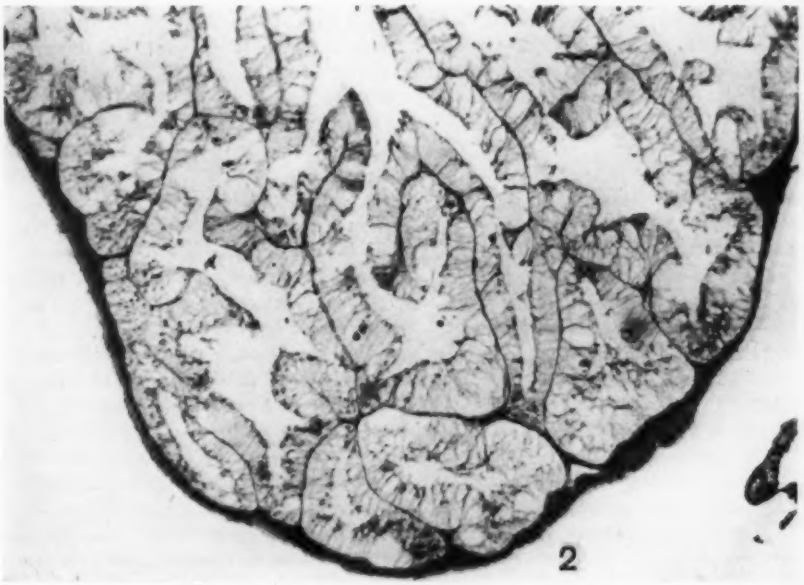

FIGURE 2. Corpus luteum of Stage *A* showing changes that take place in the granulosa soon after ovulation. × 100.

can yet be distinguished in Stage *C* where they have been reduced to small yellow structures only 3 or 4 mm. in diameter. The luteal cells of these structures have lost most of their cytoplasm and about all that remains are small cells with picnotic nuclei scattered through the shrunken connective tissue framework of the original corpus luteum.

In Stage *D* very small, compact, yellow masses of tissue can be found that seem to be all that remains of the luteal bodies. Histologically, they are composed of small cells of irregular shapes with little cytoplasm and dense nuclei loosely distributed in the connective tissue skeleton of the old corpus luteum (Fig. 6).

These observations indicate that the corpora lutea in the spiny dogfish remain in the ovary for the better part of two years and during this time they undergo a progressive decrease in size and histological degeneration.

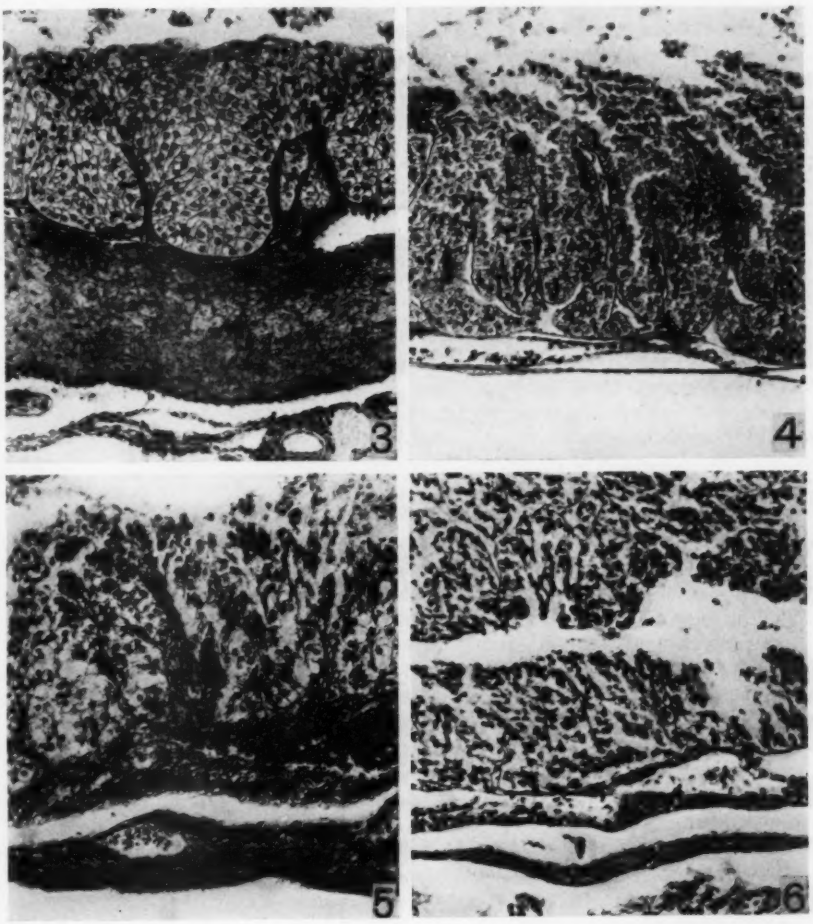

FIGURE 3. Corpus luteum of Stage *A* somewhat older than that shown in Figure 2. The granulosa luteal cells have lost their vacuoles, decreased in size and become organized into a folded multi-layered lining of the follicular cavity. × 100.

FIGURE 4. Corpus luteum of Stage *B*. The corpus luteum has decreased in size by at least one-half, and consequently the folds are more compressed. The luteal tissue is degenerating as indicated by a general decrease in size of the cells, small irregularly shaped nuclei and fraying with release of cells into the lumen.

The connective tissue inclusions between the folded granulosa luteal cells of the corpus luteum can be seen distinctly. × 100.

FIGURE 5. Corpus luteum of Stage *C*. Such corpora are about one year old and show advanced degenerative changes. × 100.

FIGURE 6. Corpus luteum of Stage *D*. Corpora of this stage are 18 to 20 months old and are reduced to small, yellow, compact bodies 2 to 3 mm. in diameter. × 100.

Nutrition of the Embryo

The embryos of *Squalus acanthias* develop free in the uterine lumen and possess no obviously specialized structures for obtaining nutritive materials from the uterine endometrium. Judging from their morphology they meet the requirements of a typical case of ovoviviparity. This and their extraordinarily long period of gestation of almost two years aroused interest in the problem of dependence or lack of dependence of the embryo on the parent as a source of nutrition. Consequently, representative material was collected from animals in the four stages of pregnancy we have described (Fig. 1) and data obtained on wet weight, dry weight, percentage of water and ash content. We have already mentioned the rather wide variations in size and weight of both ova and embryos so it is quite possible that our data are not sufficient in range and numbers to be considered as exact for the four stages of development represented, but we believe they show at least the general situation.

The ova of Stage *A* are surrounded by the thin membrane of the candle and, as they usually burst when this envelop is opened, it is extremely difficult to obtain weights of individual ova. Consequently, the whole candle was weighed, the small amount of fluid beneath the membrane at each end was withdrawn as completely as possible and the ova released into a dish by rupturing the envelop. After deducting the weight of the fluid and membrane the average weight of the ova was determined. Twenty-six candles, each containing one to four ova, when treated in this way gave an average weight of 46.2 grams per ovum. Seven ova, representing the upper limit of variation in weight, taken from two candles (3 in one and 4 in the other) of a large fish averaged 58.67 grams. This material was used for making the analyses shown in Figure 1.

In the study of Stage *B* an attempt was made to select embryos from the two extremes in the variation of development. At first, separate determinations were made for the embryo and yolk sac but for the smaller embryos the water content was so high and the amount of solids so small that it was thought a combined analysis would be better for the rather general purpose in mind. Such embryos are about 3.5 cm. long, weight 0.259 gm., and are 90 per cent water.

The pups of Stage *C* vary between 12 and 20 cm. in length, and they have yolk sacs of widely different sizes. From the standpoint of selecting material for analyses, this is complicated further by the fact that pups of the same length may have yolk sacs so different that one may be three times the weight of the other. Consequently, young that seemed to be in general representative samples of the upper and lower range in length were used for making the determinations shown in Figure 1.

Three small pups 12.5, 13, and 13.5 cm. long having an average wet weight of 11.05 gm. and dry weight of 2.25 gm. had an average total ash of 0.153 gm. Their yolk sacs averaged 21.0 gm. wet weight, 12.7 gm. dry weight and had an average ash content of 0.275 gm. The average combined ash of yolk sac and pup was 0.428 gm. A large pup 20.2 cm. long weighing 25.4 gm. wet weight, 5.8 gm. dry weight had an ash content of 0.122 gm. Its yolk sac weighed 35.1 gm. wet weight, 20.5 dry weight and had 0.331 gm. of ash. The combined weight of ash for the pup and yolk sac was 0.453 gm. A pup of about the same length (19 cm.) and weight (25.6 gm. wet weight, 5.45 gm. dry weight) but with a yolk sac only about half as large (15.8 gm. wet weight, 7.21 dry weight) had a combined ash content of 0.236 gm. (pup 0.114 gm., yolk sac 0.122 gm.).

These results have several points of interest. First the total ash of the young of this stage is not much different from that of Stage *B* although it is divided between the yolk sac and pup. Also the amount of ash is less than that found for the ova of Stage *A*. Another result that seems remarkable and is not understood is the relatively high ash content of the small pups.

The material used for the study of Stage *D* consisted of two large pups. The largest was 29 cm. long, weighed 84 gm. wet weight, 25.2 gm. dry weight, and had a total ash of 0.530 gm. The yolk sac was completely absorbed but the umbilicus was yet open (Fig. 1). The other was 24.5 cm. in length, weighed 55.5 gm. wet weight, 15.1 gm. dry weight and had a total ash of 0.3169 gm. This fish had a small yolk sac about 2 cm. in diameter (Fig. 1).

It seems obvious that the large pup was about ready to be born and equally obvious that the smaller pup never could have attained the size of the larger before parturition. Therefore there must be a wide difference in both length and weight of the young at birth. However, similar variations were found in all of the four stages of gestation that were studied, and these observations are in agreement with those made by Templeman. It seems reasonable to suspect that the size of a pup at birth may be correlated with the size of the ovum from which it came. If we make this assumption, then in our analyses, the large pups should be compared with the large ova and the small pups with the small ova. If the results for total ash are compared on this basis it is at once clear that the ash of the ovum exceeds that of the pup.

Therefore, it appears that the developing young of the spiny dogfish need not depend on the parent for inorganic matter. However, the presence of more inorganic matter in the egg than in the young at term does not preclude the possibility of a differential loss and absorption of mineral salts. Determination of the components of the ash would be helpful in answering this question but probably would not be conclusive.

Our data also indicate a loss of organic matter during development and an increase in water. When the ova of Stage *A* are compared with the mature pups of Stage D it is seen that the dry weight of the small ovum is 8.1 grams heavier than that of the small pup and the difference between the dry weights of the large ovum and large pup is 6.31 grams. The small pups also show a 75 per cent gain in water and the large pups a gain of 85.8 per cent.

These data were obtained from a few selected samples that we thought were representative of the four stages of gestation as seen at Woods Hole, Massachusetts. Therefore they should not be considered as being quantitatively exact but rather as showing the general trend of events taking place during development. It is definitely certain that the developing young obtain water from the parent, but whether they are entirely independent with regard to organic and inorganic nutrients is yet a question. However, these observations indicate that gestation in *Squalus acanthias* goes on under ovoviviparous conditions.

Discussion

Thus it is seen that the mature females caught at Woods Hole in the spring and fall are carrying young, almost without exception, and that they can be divided into four distinct groups on the basis of foetal development, size of ovarian ova and condi-

tion of the corpora lutea. These groups are so characteristic that no overlapping was found in the examination of several hundred fish. In fact it is extremely simple to determine the group to which a fish belongs by the gross appearance of the ovaries and uterine young.

It seems unmistakable that these four groups represent four stages of a gestation that extend over the better part of two years, probably 20 to 22 months. It is true that at present we can only surmise what takes place during the six months from November until May while the fish are on their southern migration. However, our records are fairly complete for the period between late April and November while these fish are in the waters along the New England coast.

When the fish arrive at Woods Hole in late April and May the embryos of the candle ova are small blastoderms and, judging by the slow rate of development, ovulation probably occurs in February or March. It is assumed that these fish (Stage A) as seen in April and May, are the same as those having candles in November (Stage *B*). Therefore, the difference between Stage *A* and Stage *B* represents the development that occurs in about the first six months of gestation, most of which is spent between Cape Cod and Newfoundland.

We also think that the fish in Stage *B* are those which, after their southern migration during the winter, return to Cape Cod the following May as fish in Stage *C*. During this period of about six months the uterine young increases in length from embryos of 3.5 to 7.5 cm. to pups of 12 to 20 cm.

These fish of Stage *C*, when next seen at Woods Hole in October and November, are in Stage *D* of gestation and a few have given birth to their young. The uterine young vary in length from about 24 to 29 cm. In some the yolk sacs are entirely withdrawn into the body while in others they are yet present but reduced to about 2 cm. in diameter. The indications are that the majority of births occur in late fall somewhere south of Woods Hole and that the pups are about 25 to 30 cm. in length when born.

Thus it seems that the only plausible explanation for these observations is that the spiny dogfish gives birth to young every other summer she visits the New England coast, and ovulates every other spring shortly before or soon after she starts on her northern migration. Furthermore, the female population is composed of two groups whose reproductive cycles are separated by about one year, and the four stages of gestation represent periods of approximately six months. Thus, each spring at Woods Hole the population includes only Stages *A* and *C* and in the fall only Stages *B* and *D*.

Our knowledge of the seasonal migration of *S. acanthias*, though fragmentary, tends to support the points just made concerning the relationships of the development of uterine young. Where the spiny dogfish spends the winter is a question that has not been answered but it is probably in deep water off the coast in the latitude of North Carolina. From there, judging from the observations of Bigelow and Welsh (1924), they arrive each spring in April almost simultaneously along the coast from Cape Lookout to Long Island. They usually appear at Woods Hole the last week of April or the first of May and are also found at about this season on Georges Bank. There seems to be a general migration northward from these points. Spiny dogfish do not appear in Massachusetts Bay before May, and Templeman (1944) reports their arrival along the coast of southern Newfoundland by

the middle of June and northern Newfoundland and Labrador by the second week of July.

Newfoundland and the southeastern coast of Labrador is the northern limit of migration. An additional point of interest is that by the time they become abundant in these waters they have almost entirely disappeared from around Cape Cod and points farther south. However, in Newfoundland, they arrive in June, become abundant in July and remain so until September or October. In contrast with this, they visit Woods Hole twice a year, in the spring and fall. Therefore, during their summer migration, which covers a period of about six months, approximately half of the time is spent in the general vicinity of Newfoundland. Judging from the observations made by Templeman the adult females are the first to arrive in the spring and apparently are the first to start south in the fall. In late November most of those caught were mature males, and immature males and females while only a few mature females were taken.

If the spiny dogfish make this round trip annually from their winter quarters, it is obvious that they must be capable of swimming great distances in a surprisingly short time. That they can do this is indicated also by Templeman's studies. He tagged 279 dogfish, mostly adult females, in the vicinity of St. John's Newfoundland between July 9th and 23rd and several were caught elsewhere in the same year. One of these, an adult female, was taken 132 days later close to Thatcher's Island Buoy, about 1,000 miles from the point where it was tagged. To travel such a distance this fish would have to average something like 7.6 miles per day even if it swam in a straight line which it is reasonable to believe it did not do. Thus it seems that at least the adult females are capable of covering what we believe is their migratory route.

A rather interesting deduction concerning migration also can be made by comparing the constitution of the population of spiny dogfish in Newfoundland and Woods Hole. Templeman states that the smallest dogfish caught was 58 cm. in length. At Woods Hole, small dogfish 40 to 50 cm. in length make up a considerable part of the general population. This indicates that these small fish, probably yearlings, do not reach the northern limit of the migratory range.

Certain differences in the stages of development of candle embryos and large uterine young of the fish examined by Templeman and ourselves also seem significant for determining migration. However, exact comparisons cannot be made as we did not make a large number of measurements that can be subjected to statistical treatment, but it is important to note extremes in size of candle embryos and large uterine young. The embryos of Stage *A* taken at Woods Hole in May were yet only blastodermic discs with an occasional exception in which a very small embryo could be seen. Of 139 comparable fish taken in Newfoundland in July, 104 had eggs showing blastodermic discs while the others had small embryos, the largest found in two animals being 1.8 and 2.0 cm. respectively. No embryos comparable to these largest ones have been seen at Woods Hole in May, and the fact that the great majority were yet blastodermic discs in July indicates that the rate of development is slow. However, in August, Templeman found only one fish in a group of 42 with eggs having blastodermic discs.

During the summer in Newfoundland growth of these candle embryos proceeds until by October and November they are from 2 to 4 cm. in length. However, by

the first of October most of the large females have left Newfoundland and it is of interest that at this time they become abundant around Cape Cod and constitute the group we have designated as being in Stage *B* of gestation. Templeman's data based on measurements of the embryos of nine fish during October and November fall well within the range of those for our Stage *B*. The only exception being that we found some embryos that were about 8 cm. in length but at the same time we had an opportunity to examine a much larger number.

Dogfish with pups having large yolk sacs (Stage *C*) taken at Woods Hole in May are comparable with those caught in Newfoundland in July. The length of the pups in Newfoundland varied from 12 to 18 cm. (average 15.59 cm.). A few at Woods Hole were 20 cm. in length, but this is probably an unimportant variation. There is also considerable variation in the size of the yolk sac. These differences in length and size of yolk sac, it seems, do not indicate necessarily a corresponding difference in age but rather is correlated with the size of the ovum at ovulation.

Such young, during the stay of the parent in Newfoundland from July to November, grew in length from an average of 15.59 cm. to 21.43 cm. The average length of the young in November is essentially the same as that for similar young taken at Woods Hole during the same month. However, there were certain interesting exceptions. The largest pups among those measured by Templeman were 25.5 cm. in length, while our largest measured 29.00 cm. The yolk sacs of these large pups had been absorbed almost completely while those of others somewhat smaller were reduced in diameter to about 1.75 to 2.0 cm. There were also a few large females with empty uteri. The large ovarian ova and uteri of these animals indicated that they had given birth to their young just recently. It seems probable that the great majority of births occur at a later time somewhere south of Woods Hole, but in a few instances young are released during the fall migration southward.

Conclusions

The spiny dogfish, *Squalus acanthias*, of the Atlantic coast migrate northward in the spring and southward in the fall. They are present at Woods Hole, Massachusetts, from late April to early June and again in October and November. The adult females when they appear in the spring, have uterine young which are either early embryos enclosed in membranous capsules ("candles") or more advanced "pups" 12 to 20 cm. in length, with large pendent yolk sacs, and free in the uterine lumen. Adult females, when seen in the fall, have candle embryos 3.5 to 7.5 cm. long or large pups 25 to 29 cm. in length, whose yolk sacs are greatly reduced or completely absorbed, while a few females have given birth to their young. Thus four distinct stages of gestation are seen when the fish are divided on the basis of development of uterine young.

The gestation period apparently covers about 20 to 22 months and a female gives birth every other year. This opinion is supported by the progressive advancement in development of the young in the four stages of pregnancy. Also, correlated with this is a gradual involution of the corpora lutea and a corresponding increase in size of the ovarian ova which will be ovulated following the conclusion of the existing pregnancy.

Analysis of candle ova and pups of the four stages of gestation show an increase in water and a decrease in both organic and inorganic matter as indicated by dry

weights and content of ash. Thus it seems that gestation in the spiny dogfish should be considered as being ovoviviparous.

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THE CULTURE OF VOLVOX AUREUS EHRENBERG

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Introduction

A number of methods for the culture of *Volvox* are described in the literature, but none of these has been consistently successful in our laboratory. Other investigators have had a similar experience. S. O. Mast (personal communication) and his students have tried these methods time and again over a period of years without securing permanent cultures.

Hartmann (1921) was unsuccessful in culturing *Volvox* although he was able to maintain cultures of *Eudorina elegans* for many months. Knoke's many experiments (1924) over a two-year period were likewise without positive results. After extensive analyses of natural waters containing *Volvox* in large numbers, Uspenski and Uspenskaja (1925) devised an inorganic salt solution (based on Knop's medium) in which *Volvox aureus* Ehrenberg and *V. globator* Linnaeus were cultured bacterium-free for periods of fifteen and four months respectively. These workers showed that a favorable concentration (0.5–1.0 mg./l.) of iron was a decisive factor in the production of healthy cultures, a deficiency of iron resulting in gradual deterioration, an excess of iron producing a poisoning of the *Volvox* colonies. Soil extract or decoction was used by Mainx (1929) who secured rich permanent cultures of *V. aureus* far superior to those obtained with synthetic solutions, including that of Uspenski and Uspenskaja. Pringsheim (1930) reached similar results in experiments with *V. globator* and *V. aureus*, but he likewise was unable to maintain these organisms for any length of time in the Uspenski medium where their growth was slower and of shorter duration than in soil extract medium. Lefévre (1932) states that he secured a clone culture of *V. aureus* in a nutritive solution (derived from that of Czurda) consisting largely of inorganic salts in pond water to which a few fragments of *Sphagnum* were added. About 300 descendant colonies were produced in some ten days, but he does not say whether this clone was maintained for any appreciable length of time. Mixed cultures of Volvocales and other plankton species, which included *V. aureus, Pandorina morum* Bory, and *Eudorina elegans* Ehrenberg, were maintained for many months.

Johansen (1940) lists six solutions which produce luxuriant growth of *Volvox, Gonium, Pandorina,* and *Eudorina,* namely certain soil solutions; 0.05 per cent Benecke's and 0.05 per cent Knop's solutions; and 1.5 per cent Detmer's agar (apparently after Bold, 1936). He also gives a medium which is identical in composition with that of Uspenski.

It would appear from the foregoing that the continuous culture of *Volvox* is a relatively simple and exact matter, and that a variety of media are available for this

[1] I gratefully acknowledge the friendly interest and encouragement in this work shown by the late Dr. S. O. Mast, emeritus professor of zoology, The Johns Hopkins University.

purpose. Nevertheless, preliminary experiments indicated that this is not the case. Soil decoctions vary widely in composition with the soil used, and inorganic salt solutions may not give rich, permanent cultures.

The purpose of the present paper is threefold: (1) to describe a culture method for *V. aureus* which has given consistent results for eighteen months in the continuous culture of the organism; (2) to reduce this method to one which is readily duplicable; and (3) to indicate by experiments the probable complexity of the nutrition of this organism.

Materials and Methods

V. aureus was secured from a pond on the property of the Carolina Biological Supply Company near Elon College, North Carolina, where it occurs throughout the year, appearing intermittently in large numbers.

In all of the experiments ordinary finger bowls (4½ inches ×2 inches) were employed as culture dishes. Two hundred cc. of medium were dispensed in each of the bowls which were then placed in a hot air oven and pasteurized. Inoculations were made after the medium had cooled to room temperature (approximately 21° C.). All cultures were illuminated by the light through a west window, some direct sunlight falling on them during part of the afternoon. Throughout the winter months the low light intensity was supplemented by radiation from a Sylvania fluorescent lamp (Type HF–150S).

Inoculations were made with ordinary medicine droppers which had been previously boiled. No attempt was made to exclude bacteria from the cultures, which however showed little evidence of bacterial action. All other possible contaminants such as protozoa and algae were absent.

The salts used in the preparation of inorganic media were either Baker's Analyzed or Merck's Reagent. The peptone and beef extract were Difco, the creatine was Pfanstiehl cp., the lactic acid was Baker's Analyzed, and the uric acid was Coleman and Bell's Reagent. In all cases stock solutions were made up and then diluted to proper concentration in the preparation of media.

The commercial fishmeal (commonly used as fertilizer) was obtained from Ballard Brothers, Willis Wharf, Virginia. The spring water used in most of the experiments was secured from a surface spring on the property of the Carolina Biological Supply Company. It is referred to as local spring water. The Huckleberry spring water came from a spring by that name near Durham, North Carolina. Distilled water was supplied by a Stokes Automatic Water Still.

Experiments and Results

Experiments with inorganic salt solutions

Modified Knop's medium as recommended by Bold (1936) and Johansen (1940) was prepared and dispensed into finger bowls. Five cultures were inoculated with several hundred colonies of *Volvox*. The cultures were examined daily, but no growth or reproduction was evident. The colonies gradually lost their rich green color and fell to the bottom of the bowl where they remained motionless, and finally deteriorated. Although the experiment was repeated a number of times the result was always the same.

Since Uspenski and Uspenskaja (1925) cultivated *V. aureus* and *V. globator* in an inorganic medium continuously over periods of fifteen and four months respectively, attempts were made to secure similar results with their medium. The composition is as follows:

KNO_3	0.025 gm.
$MgSO_4$	0.025 gm.
$Ca(NO_3)_2$	0.100 gm.
KH_2PO_4	0.025 gm.
K_2CO_3	0.0345 gm.
$Fe_2(SO_4)_3$	0.00125 gm. (added every 10 days)
Distilled water up to	1,000 cc.

Whereas Uspenski and Uspenskaja worked with bacterium-free cultures and used Leningrad glass culture dishes, the present experiments were conducted in ordinary glass finger bowls containing pasteurized medium. The initial pH was found to be 7.6 which is in exact agreement with these authors.

Five cultures were prepared. Each was inoculated with a single large *Volvox* colony bearing eight daughter colonies. Only one culture showed any evidence of growth and reproduction about a hundred pale green colonies being evident after several weeks. These were permitted to concentrate at one side of the bowl, removed with a medicine dropper, and used as inoculum for five subcultures. Observation over a period of four weeks showed no evidence of growth and reproduction. Gradually the colonies lost their motility, became paler in color, and finally disintegrated. Although the experiment was repeated a number of times, the results were negative.

It was noted that the ferric sulfate had a tendency to be precipitated in some of the cultures shortly after they were pasteurized. Believing that failure of the experiment might be due to a lack of the iron salt, it was repeated with the exception that $FeCl_3$ was used instead of $Fe_2(SO_4)_3$; but, although the former salt remained in solution somewhat better than the latter, the end result was the same.

Experiments with media containing organic materials

Soil decoctions. Several samples of local garden soils were obtained, extracted, and diluted according to the methods of Mainx (1929) and Pringsheim (1930). Although numerous attempts with varied concentrations of soil extract medium were made to secure cultures comparable to those described by these workers, success was not realized. In most instances the organisms rapidly deteriorated and died. In several cases multiplication occurred for a time, but efforts to maintain these cultures through subcultivation failed.

Peptone, beef extract, milk, and urine. Various concentrations of these substances in spring water ranging from 5 mg./l. to 200 mg./l. were tested without securing growth and reproduction for more than a week or so. The first cultures of *Volvox* in beef extract medium (5 mg./l.) exhibited an astounding rate of multiplication for several weeks, thousands of colonies being produced. Subcultures, however, were poorer. Gradually the colonies became paler in color, and after six weeks the third set of subcultures died out. Some evidence of growth was secured in peptone medium (5 to 10 mg./l) but *Volvox* colonies quickly deteriorated in all concentrations of urine and milk media, in which a considerable growth of bacteria

was evident. Even though all of the culture media contained $FeCl_3 \cdot 6H_2O$ (0.5 cc. 1 per cent sol./l.), this failed to prevent the cultures from dying out.

Commercial fishmeal. An extract of commercial fishmeal was prepared by adding 200 mg. of fishmeal to a liter of spring water and heating to 80–90° C. The mixture was shaken well and filtered (Reeve filter paper, No. 201). The slightly straw-colored filtrate was dispensed in five finger bowls which were then pasteurized. Several hundred *Volvox* were inoculated into each of these. After two weeks one of the cultures was teeming with colonies. Five subcultures from it produced thousands of the organisms. Subcultivation was continued every two weeks, but after six weeks all of the descendant cultures suddenly died out.

This experiment was repeated in exactly the same way except that the cultures were aerated daily by bubbling air through the medium, but the end result was the same.

Recalling that Uspenski and Uspenskaja (1925) had emphasized the importance of iron in the nutrition of *Volvox*, fishmeal extract was prepared as in the previous experiments. To each liter 0.5 cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ was added. This gave a concentration of the salt of 5 mg./l. or of 1.03 mg. Fe/l. This amount of Fe is slightly greater than the optimum (0.5–1.0 mg./l.) given by Uspenski and Uspenskaja, but it was felt that some of the Fe would be bound by organic substances in the medium and so reduce the available supply. The amount of Fe remaining unbound would be within the optimal range.

Five cultures were set up and inoculated from a clone culture that had been established in fishmeal extract several weeks earlier. This culture contained no iron other than that already present in the spring water used in its preparation.

In approximately two weeks excellent cultures resulted. From these cultures others were established which were in no way inferior. Thus, this clone of *Volvox* has been maintained in continuous culture with undiminished vigor for a period of eighteen months.

Following the recommendation of Uspenski and Uspenskaja, only half as much iron (¼ cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$) was added in winter as in summer. Since the initial concentration of iron was rather high, iron was not added every ten days in summer and once a month in winter as they suggested. The initial pH of the fishmeal medium was consistently 7.6. Over a three-week culture period it was found to fluctuate around this value.

Fishmeal medium, prior to the addition of ferric chloride, is faintly straw colored but clear. After twenty-four hours it becomes slightly cloudy, perhaps through bacterial action. No membrane forms at the surface, however, and in a few days the medium becomes clear.

No attempt has been made to count the total number of colonies in a single culture, but the number surely runs up to several thousand. Cultures reach a maximum population in from two to three weeks depending largely on variations in light intensity and temperature. Over a period of eighteen months reproduction has been asexual; sexual reproduction has not been observed.

Extracts of different strengths in which the amount of fishmeal varied between 50 and 1,000 mg./l. were tested, but 200 mg./l. seemed to give the largest populations of *Volvox*. Therefore, all stock cultures were maintained in media prepared with that amount of fishmeal.

Composition of fishmeal extract

Since *V. aureus* flourished in a medium prepared from spring water, fishmeal, and ferric chloride, it seemed desirable to determine the essential components with a view toward simplification. In this connection the following questions are pertinent: Can a spring water of known chemical composition be substituted for the local spring water? Can spring water be replaced by either tap water or distilled water? Can Uspenski's medium be used in place of spring water? Are the inorganic salts alone in fishmeal, along with those in spring water, sufficient to support the culture of *Volvox?* Does the fishmeal extract contain an appreciable amount of protein, and if so, how important is this in the medium? How important are other extractives?

Water component of fishmeal extract. Seven lots of five cultures were prepared in which the following media were used:

1. Fishmeal + local spring water + $FeCl_3 \cdot 6H_2O$
2. Fishmeal + Huckleberry spring water + $FeCl_3 \cdot 6H_2O$
3. Fishmeal + tap water + $FeCl_3 \cdot 6H_2O$
4. Fishmeal + distilled water + $FeCl_3 \cdot 6H_2O$
5. Fishmeal + Uspenski medium
6. Fishmeal + Uspenski medium ($FeCl_3 \cdot 6H_2O$ used instead of $Fe_2(SO_4)_3$)
7. Local spring water + $FeCl_3 \cdot 6H_2O$

In all cases 200 mg. of fishmeal and ½ cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ were used.

Each culture was inoculated with ten large *Volvox* colonies of approximately equal size, each colony containing eight daughter colonies.

Volvox flourished in media 1 and 2, but little or no growth occurred in 3, 5, 6, and 7. Large numbers of colonies were produced in medium 4. These were pale green in color, becoming paler with each subcultivation, and finally were moribund after four transfers (68 days). Cultures in media 1 and 2 were maintained for many months and were in a flourishing state when finally discarded. Repetition of the experiment gave substantially the same results.

This experiment clearly shows that the chemical composition of the water used in fishmeal medium is very important in the culture of *Volvox*. Tap water may not be nutritively deficient but toxic, for it has been observed that paramecium, hydra, and other invertebrates do not survive long in the local unchlorinated, artesian water. Distilled water is obviously deficient in certain essential salts, although the lack is not felt for some time. Huckleberry spring water can be substituted for the local spring water since it can supply these salts in adequate amounts. It is interesting to note that the Uspenski medium, prepared to include either ferric sulfate or ferric chloride, failed to support growth and reproduction of *Volvox*, even when fortified with the extractives of fishmeal. Finally, fishmeal supplies important nutritive substances without which cultures of *Volvox* soon perish.

Fishmeal component. Obviously fishmeal is a very complex material. Therefore, no extensive series of experiments was contemplated to determine what substances in fishmeal are so vital in the nutrition of *Volvox*. The following experiment was performed to make clear what class or classes of substances (inorganic

salts, proteins, other extractives) in fishmeal contribute to the growth and reproduction of *Volvox*. Four culture media were prepared according to the schema:

1. Fishmeal ash + local spring water + $FeCl_3 \cdot 6H_2O$
2. Fishmeal + cold local spring water + $FeCl_3 \cdot 6H_2O$
3. Fishmeal residue + hot local spring water + $FeCl_3 \cdot 6H_2O$
4. Washed fishmeal + local spring water + $FeCl_3 \cdot 6H_2O$

One gram of fishmeal was completely incinerated in a chemically clean crucible and the resulting ash shaken well with one liter of spring water. The mixture was heated to 80–90° C. and filtered while hot. Two hundred cc. of this solution were diluted to one liter with spring water in order to secure an inorganic salt concentration comparable to that of regular fishmeal extract.

Another gram of fishmeal was added to a liter of spring water, shaken well, and filtered. Two hundred cc. of the filtrate were diluted with spring water to one liter to give a solution of cold-water extractives approximating the concentration of these substances in regular fishmeal extract.

The residue on the filter paper was added to a liter of spring water and heated to 80–90° C. and filtered while hot. Two hundred cc. of the filtrate were diluted with spring water to one liter. Theoretically this solution contained hot-water extractives roughly equivalent to their concentration in regular fishmeal extract.

The residue on the filter paper was dried and weighed. It had lost one-fourth of its weight. This amount (0.25 gm.) was shaken well with a liter of spring water to form a suspension.

One-half cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ was added to each liter of culture medium. Five cultures were prepared from each of the four media, pasteurized, and allowed to cool to room temperature. Each culture was inoculated with five large *Volvox* colonies, every one of which contained eight daughter colonies in about the same state of development.

Examination of the cultures over a period of three weeks gave no evidence of growth in media 1, 3, and 4. Medium 2 on the other hand gave excellent cultures which were in no respect inferior to stock cultures maintained on regular fishmeal extract. Subcultures, which were carried for several months, showed no sign of deterioration whatsoever and probably could have been maintained indefinitely.

This experiment demonstrates, therefore, that the readily soluble cold-water extractives of fishmeal complement the inorganic salts of spring water and the ferric chloride to produce a nutritionally complete medium; that the soluble salts in fishmeal ash medium do not meet all of the growth requirements of *Volvox;* and that hot-water extractives and proteins of fishmeal have little or no importance in the culture of *Volvox*.

It might be supposed that proteins would be present in the cold-water extract fraction of fishmeal, but the usual tests for protein were negative.

Fish extracts

Commercial fishmeal consists largely of the pulverized remains of marine fishes, and therefore contains all of the chemical constituents of bone, muscle, skin, viscera, etc. The question as to what part of a fish, if any one part, provided the important nutritive substances in the culture of *Volvox* was investigated as follows:

Several butter-fish (*Poronotus triacanthus* Peck) were secured at a local fish market. These were washed in distilled water. Small quantities (about 10 gm.) of skin, muscle, and bone (vertebral column) were removed very carefully in order not to include any of the adjacent tissues. The three portions were placed in a small amount of distilled water in three separate flasks and boiled until practically all of the water had evaporated. They were then dried on filter paper in a dry air oven. One-half of each portion was separately shaken with ether from time to time for a period of four hours in order to remove lipoids, after which treatment the ether was removed by filtration. The skin, muscle, and bone residues were dried on filter paper for forty-eight hours. The remaining half of each portion of skin, muscle, and bone received no further treatment.

Two hundred mg. each of skin, skin extracted with ether, muscle, muscle extracted with ether, bone, and bone extracted with ether were separately heated with one liter of spring water to 80 – 90° C. and filtered while hot. One-half cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ was added to each liter of filtrate. Five cultures were prepared from each type of filtrate and inoculated with numerous *Volvox*.

The results are summarized below:

1. Skin medium—colonies died within a few days
2. Extracted skin medium—colonies died within a few days
3. Muscle medium—good cultures; subcultured for three months
4. Extracted muscle medium—excellent cultures; subcultured for three months
5. Bone medium—colonies died within three weeks
6. Extracted bone medium—some growth and reproduction; subcultures unsuccessful

The experiment was repeated using Huckleberry spring water instead of the local spring water. The results were essentially the same with the exception that colonies in the extracted bone medium died out within a week.

The experiment shows that, of the three tissues tested, muscle alone contains an adequate quantity of the substances so vital to the continuous culture of *Volvox*. Furthermore, the conclusion is reached that the lipoids are of no value to *Volvox;* on the contrary they appear to exert a depressing influence as is indicated by the larger populations of extracted muscle medium cultures.

No attempt is made here to deny that connective tissue associated with fish muscle may contribute as much or more than muscle to the nutrition of *Volvox*. It would be next to impossible to separate the two tissues to determine this point. However, in experiments which follow the assumption is made that it is the muscle that is important and not the connective tissue.

Since the butterfish is marine and the associated salts of sea water might conceivably have something to do with the results obtained, experiments were carried out with the fresh-water sunfish (*Lepomis gibbosus* Linnaeus) in order to check this presumption. The ether-extracted, powdered muscle gave flourishing cultures which were maintained in subculture for three months and then discontinued. Doubtless muscle tissue from other fishes could have been used with similar results.

Experiments with some organic constituents of fish muscle

Whatever the nature of the constituents of fish muscle that are so important in the culture of *Volvox*, it is clear from the foregoing experiments that the proteins,

lipoids, and inorganic salts are unnecessary. This leaves carbohydrates (glycogen, etc.), nitrogenous extractives (creatine, urea, uric acid, etc.), non-nitrogenous extractives (lactic acid, inosite, etc.), pigments, enzymes, and growth-promoting factors, and perhaps other substances to be considered. It seemed most likely that one or more of the nitrogenous and non-nitrogenous extractives might support the continuous culture of *Volvox*. Therefore, experiments were undertaken in which lactic acid, urea, creatine, and uric acid were tested singly and in combination in various concentrations from 5 mg./l. to 100 mg./l. of local spring water, the usual amount of ferric chloride being added to all media. None of the cultures, however, was successful. In several instances adult colonies released daughter colonies, but these gradually lost their healthy green color and soon deteriorated. In a medium composed of urea, peptone, and lactic acid (5 mg./l. each) as many as three generations were produced before death occurred.

A duplicable culture medium for Volvox aureus

An analysis of Huckleberry spring water by D. M. Pace some fifteen years ago (personal communication) showed it to have the following inorganic composition:

Na_2SiO_3	12 mg./l.
NaCl	12 mg./l.
Na_2SO_4	6 mg./l.
$CaCl_2$	6.5 mg./l.
$MgCl_2$	3.5 mg./l.
$FeCl_3$	2–3 mg./l.

An artificial spring water was made up according to the above formula except that sufficient ferric chloride solution was added to give a concentration of this salt of 5 mg./l. Using ether-extracted, powdered sunfish muscle (200 mg./l.), culture medium was prepared according to the same method previously described for the fishmeal and fish muscle media. Cultures of *Volvox* have been maintained on this medium in a flourishing state for a period of four months, showing no evidence of decrease in vitality. These cultures were in every respect equal to cultures of the organism secured with commercial fishmeal extract.

Although fishmeals may vary somewhat in composition depending on their source and mode of manufacture, the powdered sunfish muscle prepared as herein described may be expected to have a constant composition wherever prepared. This method for the culture of *V. aureus*, therefore, is readily duplicable.

Discussion

Results of the foregoing experiments fail to confirm those of Uspenski and Uspenskaja (1925), Bold (1936), and Johansen (1940) who have cultured *Volvox* in inorganic salt solutions in the absence of organic matter. The findings, however, are in agreement with those of S. O. Mast (personal communication) and his students who were unable after many experiments to secure permanent cultures of *Volvox* in Uspenski's, Knop's, and other synthetic media. Pringsheim (1930) was evidently unable to maintain *V. aureus* and *V. globator* for any length of time in the Uspenski medium.

It is difficult to believe that failure to culture *Volvox* in the Uspenski medium was a result of impurities in the salts or the kind of glassware used in the present experiments. The salts were of the same degree of purity as those generally employed in the culture of protozoa and algae. The salts used in the preparation of artificial Huckleberry spring water apparently had no toxic effect.

Various investigators, notably Pringsheim (1930), have called attention to the deleterious effects produced on organisms by the kind of glass of which culture dishes are composed. He believed that unfavorable results were due to soluble constituents of the glass causing the medium to become more alkaline. Although ordinary finger bowls were used in the present experiments, no deleterious effect of the glass was observed. Successful cultures on fishmeal and fish muscle extract media were secured in the same glass finger bowls in which Uspenski's medium failed to support continuous growth and reproduction of *Volvox*.

Nor can failure to secure permanent cultures in the Uspenski medium be ascribed to an unfavorable pH. The initial pH was 7.6 (identical with that given by Uspenski and Uspenskaja) and remained at that value over a period of three weeks. That this is a favorable reaction is shown by the fact that the pH of freshly prepared fishmeal extract medium is 7.5–7.6, around which value it fluctuates from day to day gradually rising to a final pH of 8.0–8.2.

Mainx (1929) and Pringsheim (1930) secured rich cultures of *Volvox* in decoctions prepared from garden soil. Soils, however, vary widely in composition and reaction so that this method is not readily duplicable. Fishmeal medium, on the other hand, can be made up fairly accurately and is more readily reproducible. This is even more true of the fish muscle extract prepared with artificial Huckleberry spring water and the muscular tissue of the sunfish or butterfish.

The experiments of the present work show that the nutritional requirements of *Volvox aureus* may be more complex than is indicated by the work of Uspenski and Uspenskaja (1925) and that inorganic salts of spring water as well as organic substances of fish muscle are essential if this organism is to be maintained in culture for any length of time. What these salts and organic substances are, remains for further work to show; but it is quite possible that water-soluble growth promoting substances play an important rôle.

Summary

1. *Volvox aureus* Ehrenberg has been maintained in rich clone cultures for eighteen months without observable decrease in vitality on a medium prepared from spring water, commercial fishmeal, and ferric chloride.

2. The presence of iron in the medium was found to be essential to the continuous culture of *Volvox*. This was added as ferric chloride. Cultures lacking iron died out in about six weeks.

3. Either local spring water or Huckleberry spring water served satisfactorily in the preparation of fishmeal extract medium, but tap water and distilled water could not be substituted for these.

4. Spring water media containing peptone, beef extract, milk, or urine in various concentrations would not support continuous growth and reproduction of *Volvox*.

5. Uspenski's medium and modified Knop's solution were found to be of little value in the culture of *Volvox*.

6. Powdered muscle of the marine butterfish (*Poronotus triacanthus* Peck) or the fresh-water sunfish (*Lepomis gibbosus* Linnaeus), with lipoids extracted, could be substituted for commercial fishmeal, while skin and bone could not.

7. Spring water containing only the inorganic salts of fishmeal, or only the proteins, failed to support the culture of *V. aureus;* but spring water containing the cold water extractives and ferric chloride (one-half cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$/l.) gave excellent cultures which probably could have been carried indefinitely.

8. *V. aureus* could not be cultured in media containing lactic acid, urea, creatine, uric acid, or a combination of urea, peptone, and lactic acid in the several concentrations used.

9. A readily duplicable method for the culture of *V. aureus* is described.

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OXYGEN CONSUMPTION AND CARBON DIOXIDE ELIMINATION IN TETRAHYMENA GELEII FURGASON [1]

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Unicellular organisms make excellent material for the study of various cellular phenomena. *Tetrahymena geleii*, a colorless holotrichous ciliate, is an exceptionally desirable organism for such physiological studies, mainly because it is readily grown in rather simple, sterile organic media.

This organism contains cytochromes c, b, a, and possibly a_2 and its oxygen consumption is inhibited by cyanide and carbon monoxide (Baker and Baumberger, 1941). It grows well at ordinary oxygen tensions, but is most prolific in pure oxygen (Pace and Ireland, 1945). It soon dies in oxygen tensions below 10 mm. Hg partial pressure.

Many investigations have been carried out with *Tetrahymena* as the experimental organism. There is, however, much to be desired in respect to our knowledge concerning respiratory metabolism; knowledge that may lead to a better understanding of the respiratory mechanisms of cells in general. Since very little is known concerning oxygen consumption, carbon dioxide elimination, and respiratory quotient in *Tetrahymena*, the following investigations were made.

Material and Methods

The "W" strain of *Tetrahymena geleii*, kindly furnished by Professor George Kidder, used throughout these investigations, was grown in this laboratory in a 2 per cent proteose-peptone (Difco) solution. They grow very rapidly in this solution and usually reach maximum numbers within four to six days depending upon temperature and the number introduced.

A Barcroft-Warburg respirometer was used for ascertaining oxygen consumption and carbon dioxide elimination. Temperatures below room temperature were obtained in the bath by means of an Aminco refrigerating unit.[2]

Before each experiment the organisms were washed thoroughly in a buffered solution in which they were left during the experiment. This solution is one similar to that used by Pace and Belda (1944) for *Pelomyxa*, with slight modifications,[3] and was used for these tests chiefly because of the several advantages it has over the proteose-peptone solution. It is much simpler to work with since there is no

[1] Aided by a grant from the Penrose Fund of the American Philosphical Society.

[2] An extra set of manometers and shaking device for the Warburg outfit was purcheasd for this investigation by a grant-in-aid furnished by the University of Nebraska Research Council.

[3] The buffer solution used for washing and testing *Tetrahymena geleii* in the Barcroft-Warburg respirometer contained the following: $K_2HPO_4 \cdot 3H_2O$, 65.5 mg.; $NaH_2PO_4 \cdot H_2O$, 40.0 mg.; $CaCl_2$, 100 mg.; $MgCl_2$, 2.0 mg.; and redistilled H_2O to 1,000 ml.

food material on which bacteria can grow and therefore the dangers of contamination are minimized. Bacteriological technique was used throughout.

Usually, for each condition tested, two different ages of cultures were used: "young" cultures (three or four days) and "old" cultures (seven or eight days). These cultures were started by introducing several thousand organisms by means of a platinum wire loop into 70 ml. of sterile proteose-peptone solution in a 125 cc. pyrex erlenmeyer flask.

After washing and after proper dilution or concentration (by centrifugation) 5 ml. of culture solution of known population density were added to each Barcroft-Warburg flask except to the thermobarometer which contained 5 ml. of buffered solution without organisms.

Oxygen consumption and CO_2 elimination were determined by the direct method. For this purpose 0.2 ml. 10 per cent KOH was put into the inner wells (insets) of one-half the flasks and 0.2 ml. H_2O in the other one-half. To each of the onsets was added 0.3 ml. 3N H_2SO_4 to absorb any ammonia that might possibly be formed in metabolism. In every case this acid was dumped into the main compartment of the flask at the end of an experiment after which a reading was always made in order to account for the bound CO_2 which is released by the action of acid.

Results

Relation between population density and O_2 consumption and CO_2 elimination

Tetrahymenas were obtained from cultures of different ages. "Young" cultures were produced in the following manner: about $2{,}000 \pm 200$ tetrahymenas were added to 70 cc. 2 per cent sterile proteose-peptone solution in pyrex erlenmeyer flasks. These cultures were kept at room temperature ($24° \pm 2°$ C.) for 3 or 4 days when the organisms were washed in the buffer solution [3] by centrifugation. They were then ready for testing. Several different population densities were used, ranging from approximately 10,000 to 195,000 organisms per ml. Several experiments were conducted for each density.

In these experiments 5 ml. of fluid containing young tetrahymenas were added to each of six Barcroft-Warburg flasks (3 with KOH in inner well; 3 with H_2O). Observations and readings were made from time to time during the course of the experiment. Usually readings were made every hour, but in the greater population densities, they had to be made every 15 or 30 minutes; whereas, with some of the lower population densities several hours elapsed in some cases before a final reading was made. It depended entirely upon the rapidity of oxygen consumption at 25° C. After the reading was made at the end of an experiment, the acid in the side arm of each flask was dumped into the fluid containing the tetrahymenas. The shaking apparatus was turned on for 10 minutes, after which time another reading was made. The results of these tests are presented in Table I.

The oxygen consumption per organism, when young cultures were used, is greatest in densities of 13,000 to 33,000 organisms per ml. which were the lowest densities used, and least in the greatest densities. In other words, at least within these limits, oxygen consumption per organism is inversely proportional to population density. The respiratory quotient varied from 0.95 to 1.13 in all the different tests, except those in which the lowest population densities were used where the R.Q. averaged 1.41.

The "old" organisms were produced in the same way as the "young" except that they were not used until 7 or 8 days after starting the cultures. The same procedures were followed as in the previous tests. The population densities varied between 12,000 and 265,000 organisms per ml. These results are also presented in Table I.

TABLE I

Oxygen consumption, carbon dioxide elimination and respiratory quotient in "young" and "old" cultures of Tetrahymena geleii *with different population densities. Temperature 25° C.; average volume of one million organisms = 23.6 mm.³; 42,370 organisms equal to one cubic millimeter.*

No. of organisms per ml.	Age of culture in days	No. of tests	O_2 consumption in mm.³ per hr. per million organisms	O_2 consumption in mm.³ per hr. per mm.³ cell substance	CO_2 elimination in mm.³ per hr. per million organisms	CO_2 elimination in mm.³ per hr. per mm.³ cell substance	R.Q.
Organisms from "young" cultures							
13,000–19,000	3	6	372	15.7	526	22.3	1.41
30,000–33,000	3	9	372	15.7	356	15.0	0.95
41,500–51,500	4	6	325	13.7	350	14.8	1.07
88,000–98,000	3	6	322	13.6	328	13.9	1.01
107,600–121,000	3½–4	9	252	10.6	249	10.5	0.99
195,000	3½	3	246	10.4	278	11.7	1.13
Organisms from "old" cultures							
12,000–12,600	7–8	9	198	8.4	221	9.3	1.11
24,000–27,500	7–8	6	223	9.4	258	10.9	1.15
38,000–39,400	7–8	9	204	8.6	247	10.4	1.21
69,000–72,600	7	6	278	11.7	357	15.1	1.28
87,600–95,000	7–8	6	217	9.2	263	11.1	1.21
135,000–148,700	7	9	207	8.7	240	10.1	1.15
213,000–265,000	7	6	230	9.7	272	11.5	1.18

The oxygen consumption varies from one population density to another much more so than in the "young" cultures, but there is a definite increase in consumption up to approximately 70,000 organisms per ml. and then a decrease with further increase in numbers per unit volume. Carbon dioxide elimination was also ascertained and the R.Q. was found to be greater than unity in every case where old cultures were used. The R.Q. increased progressively from 1.11 when approximately 12,000 organisms per cc. are used, to 1.28 when approximately 70,000 are used. It then decreases with further increase in cell population until it is 1.18 in cultures containing between 213,000 and 265,000 organisms per ml.

Relation between temperature and oxygen consumption

Tests were made to ascertain the oxygen consumption and carbon dioxide elimination in Tetrahymena at different temperatures. The temperatures used varied from 10° to 35° C. (with 5° increments). As in the previous experiments tests were run on both "young" and "old" organisms. The results are given in Table II.

There is an increase in oxygen consumption as temperatures increase up to 25° C. Above 25° C. the O_2 consumption decreases directly with increase in temperature. This applies to organisms obtained from both young and old cultures. Attempts were made to ascertain oxygen consumption at 40° C. but the organisms died at this temperature. The carbon dioxide elimination and hence the R.Q. values were high in all the tests made. The latter ranged between 1.05 and 1.39.

TABLE II

The effect of temperature on oxygen consumption, carbon dioxide elimination and respiratory quotient in Tetrahymena geleii. *Average volume of one million organisms, 23.6 mm.³; 42,370 organisms equal to one cubic millimeter.*

Temperature, ° C.	No. of organisms per ml.	Age of culture in days	No. of tests	O_2 consumption in mm.³ per hr. per million organisms	O_2 consumption in mm.³ per hr. per mm.³ cell substance	CO_2 elimination in mm.³ per hr. per million organisms	CO_2 elimination in mm.³ per hr. per mm.³ cell substance	R.Q.
Organisms from "young" cultures								
10	31,800	3	3	50	2.1	57	2.4	1.14
15	52,900–67,500	3–3½	9	125	5.3	142	6.0	1.13
20	38,750–41,160	3	6	193	8.1	227	9.6	1.17
25	41,500–51,600	4	6	325	13.7	350	14.8	1.07
30	32,000–60,000	3	6	227	9.6	297	12.5	1.30
35	10,000	3	3	215	9.1	287	12.1	1.33
Organisms from "old" cultures								
10	54,000	8	3	25	1.0	35	1.5	1.39
15	53,000–73,300	7	12	92	3.9	91	3.8	0.99
20	65,500	6–7	6	128	5.4	135	5.7	1.05
25	69,000–72,600	7	6	278	11.7	357	15.1	1.28
30	45,000–50,800	7	6	228	9.6	285	12.0	1.25
35	16,800–19,160	8	6	254	10.7	287	12.1	1.12

O_2 consumption and CO_2 elimination in Tetrahymena in proteose-peptone solution

All the previous tests which have been reported here were made on Tetrahymena in the buffer solution given in footnote 3. None had been made on organisms in solutions in which they had grown and lived, i.e., proteose-peptone solution. The following experiment was carried out in order to make these determinations. Thus, the tests were conducted in the same way as the preceding ones, except that the organisms (1) were kept in the solution in which they had grown (2 per cent proteose-peptone) or (2) were washed in fresh proteose-peptone and then put into the manometer flasks in this solution. It was found that the hydrogen-ion concentration did not hold to a constant value as well as in the buffer solution but the change did not seem to harm the organisms (the greatest change noted was a change from pH 6.8 to pH 7.3). The results are presented in Table III.

In the tests in which "young" cultures of Tetrahymena were used in fresh proteose-peptone solution, the oxygen consumption proved to be much greater than in those tested in buffer solution (at 15° C., 271 mm.³ compared to 125 mm.³ per hour

per million organisms in buffer solution; at 25° C., 666 mm.^{3} compared to 325 mm.^{3}). In the organisms from "old" cultures, however, the oxygen consumption was somewhat lower than that in buffer solution (at 15°, 70 mm.^{3} as compared to 92 mm.^{3}). Variance in numbers, however, must be taken into consideration.

TABLE III

Oxygen consumption, carbon dioxide elimination and respiratory quotient in Tetrahymena geleii *in 2 per cent proteose-peptone solution. Temperature varied as indicated; average volume of one million organisms, 23.6 mm.^{3}; 42,370 organisms equal to one cubic millimeter.*

Temperature, °C.	No. of organisms per ml.	Age of culture in days	Condition of proteose-peptone solution	No. of tests	O_2 consumption in mm.^{3} per hr. per million organisms	O_2 consumption in mm.^{3} per hr. per mm.^{3} cell substance	CO_2 elimination in mm.^{3} per hr. per million organisms	CO_2 elimination in mm.^{3} per hr. per mm.^{3} cell substance	R.Q.
15	30,400	3	fresh	4	271	11.4	328	13.9	1.21
15	32,060–40,000	7	old	6	70	2.9	197	8.3	2.81
25	25,800	3	fresh	3	666	28.2	849	35.9	1.27

A very interesting observation brought out by these results, is that in all tests, the R.Q. is considerably above 1.0. This is especially so in the case of the "old" organisms where the average R.Q. for all tests was 2.81.

Significance of the high respiratory quotients

The question arises as to the true meaning of these high respiratory quotients. In all but a few experiments covered by this report, they are greater than 1.0. It is generally known that high values for R.Q. may be obtained chiefly under two conditions: (1) when fat is in the process of formation in cells or tissues, or (2) when it is necessary for the cell or tissue to go into oxygen debt, temporarily at least, during which time the ratio between CO_2 eliminated and O_2 consumed would be much greater than usual.

Tetrahymena produces fat in the form of small globules located in the anterior half of the organism. Observations were made upon these organisms under various conditions in order to ascertain whether or not respiratory quotients varied and if so, whether or not fat content varied also. As far as could be seen by the methods used there was no difference in fat content in organisms under different conditions even though the respiratory quotients varied considerably.

A few tests were made in an attempt to ascertain the oxygen consumption and carbon dioxide elimination of *Tetrahymena* in nitrogen. The nitrogen used, however, was a commercial grade and hence traces of oxygen were present. Although the results obtained by these tests were not conclusive, they indicate that these organisms are anaerobic, at least to a limited extent, thus confirming the results of Thomas (1942). The respiratory quotients calculated from the results ranged between 1.49 and 2.87.

DISCUSSION

Hall (1938, 1941), Baker and Baumberger (1941) and Ormsbee (1942) have studied respiration in *Tetrahymena geleii*. The results of these several investiga-

tions appear to differ, in some cases, considerably but when they are analyzed carefully the differences, for the most part, may be explained.

Some of the results of the present investigation are in close agreement with those of the aforementioned workers. This is especially true, when our results are compared with those of Ormsbee (1942) in which he found that the O_2 consumption for these organisms in 2 per cent proteose-peptone solution is 632.5 mm.^{3} per hour per million at 26.8° C.; in our studies, it was found that the O_2 consumption in 2 per cent proteose-peptone is 666 mm.^{3} at 25° C. The results obtained in non-nutrient media should not be compared since the salts differ greatly in the solutions used by different investigators, but it is found that even in these cases there is some agreement, especially when all the factors are taken into consideration. An explanation of some of the differences, for example, might be found in the fact that different population densities were used. According to our results, as the density of population increases the rate of oxygen consumption per individual decreases. This observation, along with possible differences in age of cultures, and also the difference in culture media before and during the experiment, could account for the slight difference in oxygen consumption as noted in these reports. This age difference is especially noticeable when the organisms are tested in the proteose-peptone solution in which they were grown. There is not only an inhibition of division with age but also a great decrease in oxidative metabolism.

Thomas (1942) showed definitely that *Tetrahymena* is adapted, at least partially, to an anaerobic existence, recovery being dependent upon oxidative metabolism. He believes the processes involved may be similar to those of mammalian striated muscle. Pace and Ireland (1945) showed that *Tetrahymena* can live and grow at very low oxygen tensions but that its growth is best at high tensions. This organism does not grow or live for any great length of time in the total absence of oxygen. Results presented in this report confirm those of Thomas in this respect.

The respiratory quotients obtained for *Tetrahymena* were all high under the conditions in which they were determined. They were highest of all in those organisms confined in practically pure nitrogen. Carbon dioxide is produced in large quantities. It is possible that the metabolism is similar to that of vertebrate striated muscle. The extent of anaerobiosis is limited for *Tetrahymena* just as it is for muscle. *Tetrahymena* can live for several days in the absence of oxygen but soon dies unless oxygen is added. Specht (1934) found the same to be true for *Spirostomum*. He obtained high R.Q. values when this organism was exposed to low O_2 tensions. Many protozoan forms function in the same way (von Brand, 1946).

Summary

1. Oxygen consumption and carbon dioxide elimination of *Tetrahymena geleii* has been ascertained for different temperatures, for different population densities, and for "young" and "old" cultures.

2. The tests were conducted in most of the experiments with washed organisms in inorganic buffer solution.

3. When "young" organisms are used, the oxygen consumption per unit volume of cell substance is inversely proportional to population density. The respiratory quotients for all tests were nearly always above 1.0.

4. When "old" organisms are used there is an increase in O_2 consumption per unit volume of cell substance with an increase in population density up to 69,000-

72,600 organisms per ml. Densities above this result in lower consumptions. Respiratory quotients varied from an average of 1.11 in low population densities to 1.28 at optimum densities.

5. Oxygen consumption is always greater in the "young" cultures than in the "old" cultures, densities being equal.

6. With an increase in temperature, the O_2 consumption increases to a maximum at 25° C. in both "young" and "old" cultures. Above this temperature the consumption decreases. In all tests, from 10 to 35° C., R.Q. values were above 1.0.

7. "Young" organisms, tested in fresh solution of the same kind in which they were grown, 2 per cent proteose-peptone (Difco), show much greater O_2 consumption than those tested in inorganic buffer solution. "Old" organisms, however, tested in the same solution in which they had grown, have a lower O_2 consumption than old specimens tested in fresh inorganic buffer solution.

8. Very high respiratory quotients were obtained for the organisms in proteose-peptone, especially in "old" cultures where the average was 2.81.

9. In nitrogen gas with but minute traces of oxygen, *Tetrahymena* utilizes the small quantity present and gives off comparatively large quantities of carbon dioxide. The R.Q. values are extremely high.

10. *Tetrahymena geleii* is to a limited extent anaerobic but it can live only with difficulty for more than a few days in the absence of oxygen.

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INFLUENCE OF TEMPERATURE ON THE ASPHYXIATION OF YOUNG GOLDFISH (CARASSIUS AURATUS L.) UNDER VARIOUS TENSIONS OF OXYGEN AND CARBON DIOXIDE

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Introduction

In their preliminary work on the sensitivity of Ontario fishes to carbon dioxide as measured by the asphyxial tension of oxygen of fish in sealed jars containing various concentrations of dissolved carbon dioxide, Fry and Black (1938) observed a difference in the response of two sample groups of the minnow, *Chrosomus eos*. The group taken in the autumn from a cold stream near Toronto was found to be appreciably more sensitive than the group from a bog lake in Algonquin Park at the height of the summer warming. These data were subsequently published in a review (Fry, 1939). At that time there was no indication as to the cause of this difference since the two groups came not only from different parts of the country but also from different habitats.

Further measurements were made in Algonquin Park in 1939 when the work was continued into early fall. Some comparisons were made of curves showing sensitivity to carbon dioxide for the same species from the same locality at different times of year. The same effect was noted as for *Chrosomus eos*, although the data were rather scanty. Observations of the same sort were made again in 1940 and, in particular, material was gathered for the brown bullhead (*Ameiurus nebulosus*). The results are illustrated in Figure 1. There is an increased sensitivity in late summer or early fall when the water is cooling. These tests were carried out in water of the same temperature as that in which the animals were captured.

Reasoning by analogy from the fact that cold water species such as the trout fail to take up oxygen in the presence of much lower tensions of carbon dioxide than do warm water species such as the brown bullhead, it was concluded that probably there would be some such trend within each species and that concomitant with the process of thermal adaptation there might be a change in respiratory sensitivity in the direction observed.

Meanwhile an opportunity to test this experimentally had presented itself in February 1939, when two of the authors in collaboration with Professor Laurence Irving and using the facilities of the experimental hatchery at Cornell University, measured the respiratory sensitivity of the speckled trout at 1° C, the temperature of the hatchery water at that time. The respiratory sensitivity was also measured of a group of these fish which had spent 24 hours at 13° C. The sensitivity of the group subjected to the warm water for even that short period of time was appreciably decreased.

This experiment, however, was only of a preliminary nature and at that time it was not possible to carry it to its logical conclusion. For this reason we undertook to do a further series of experiments to determine the effect of temperature on the respiratory sensitivity of thermally adapted goldfish. The temperatures chosen covered the biokinetic range of temperature for this species. Goldfish were maintained at each temperature level for a period of time quite sufficient to ensure a thorough thermal adaptation. The goldfish was chosen because of its suitability as a laboratory animal, because the thermal tolerance for the young goldfish had been

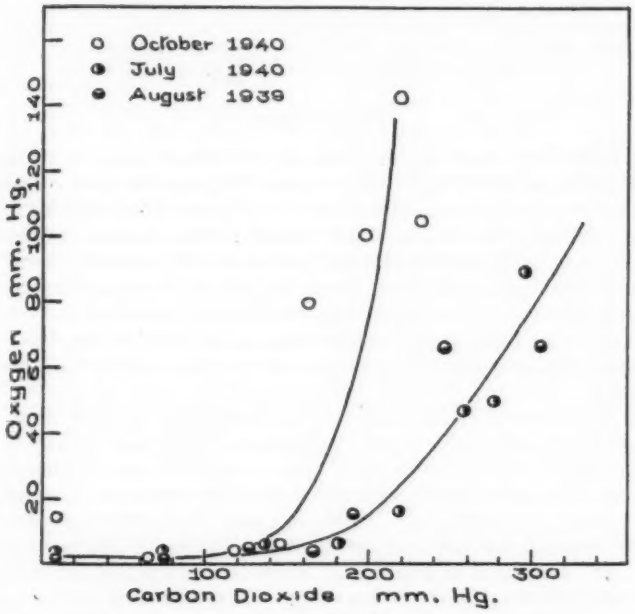

FIGURE 1. Seasonal variation in the extent to which the brown bullhead can reduce the oxygen content of water in sealed bottles in the presence of various tensions of carbon dioxide. In July and August the fish were acclimated to approximately 20° C., in October to 13° C. They were tested at the acclimation temperatures in each instance.

plotted with some exactitude (Fry *et al.*, 1942), and also because the rate of thermal adaptation of goldfish was being measured (Brett, 1946) at the time these experiments were begun.

MATERIAL AND METHODS

The goldfish, *Carassius auratus* (Linnaeus), were the ordinary long finned commercial variety of goldfish purchased from commercial dealers in Ontario. They were approximately one year old, of both sexes, and varied from 2½ to 4 inches in length. The fish were fed fox chow during the period of acclimation.

A group of fish was acclimatized to one of the following temperatures: 1° C., 7° C., 15° C., 20° C., 25° C., and 32° C. The overall allowance for acclimation of each

group of fish from 7° to 32° was 1° C. or less per day until the desired temperature was reached. Since complete acclimation to low temperatures takes a longer time (Doudoroff, 1942) the fish used for the 1° C. curve were put from a 7° C. tank into a 1° C. tank and left for two months.

When acclimation was complete each fish was put in a bottle of water (275 ml. capacity) of the same temperature and containing a known tension of carbon dioxide and at least 150 mm. tension of oxygen. During the experiment the sealed bottles containing the fish were kept in a water bath at the temperature of acclimation. When all respiratory movements of the fish had ceased the water in the bottle was analyzed for free carbon dioxide and oxygen. The tension of carbon dioxide was determined as follows: a small bubble of air (0.1–0.3 ml.) was equilibrated with 35 ml. of the water for five or more minutes at the temperature of the experiment at room pressure; the bubble was then transferred to a micro-gas-analyzer (after Krogh, 1908) and the carbon dioxide content of the gas phase was found by absorption of carbon dioxide with ¼N potassium hydroxide. The tension was obtained by multiplying the fraction of carbon dioxide in the gas phase by the barometric pressure minus the vapor pressure of the water at the temperature of the experiment.

Dissolved oxygen was determined by the unmodified Winkler method, using a 50 ml. sample of water. The pressure of oxygen was calculated by relating the quantity of oxygen found to the solubility at the temperature of the experiment and multiplying the fraction obtained by 760.

Results

The data for the experiments at each of the six temperatures (1° C., 7° C., 15° C., 20° C., 25° C., and 32° C.) are given in Table I and illustrated in Figure 2. To

Table I

The extent to which goldfish acclimated to various temperatures can reduce the oxygen content of water containing dissolved carbon dioxide. Each pair of values is the mean of three to five determinations

1° C.		7° C.		15° C.		20° C.		25° C.		32° C.	
CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg
20	5.5	27	3.5	17	2.9	34	7.5	25	4.7	14	8.0
64	4.9	79	3.5	66	3.5	88	8.3	141	13	144	8.0
87	48	100	5.8	110	5.9	120	2.9	172	7.6	168	7.0
98	24	113	16	131	12	139	7.3	200	42	186	7.3
111	46	128	38	144	49	154	41	224	120	212	46
141	66	138	66	153	49	166	25	286	272	226	102
				171	74	197	80			240	216
				192	75	242	139				
				237	163						

reduce the spread the original figures have been grouped into averages of 3 to 5 determinations. This averaging was carried out by arranging all the values in a particular series in order of the magnitude of the carbon dioxide tension and then averaging the values for consecutive groups.

The course at each temperature appears to be as follows. Up to a certain tension of carbon dioxide the oxygen in the bottle is reduced to a uniform and low level before death of the fish ensues. Above a certain carbon dioxide tension the level of the carbon dioxide influences the level to which the oxygen can be reduced, i.e., the greater the carbon dioxide content the higher the level of oxygen in the water at

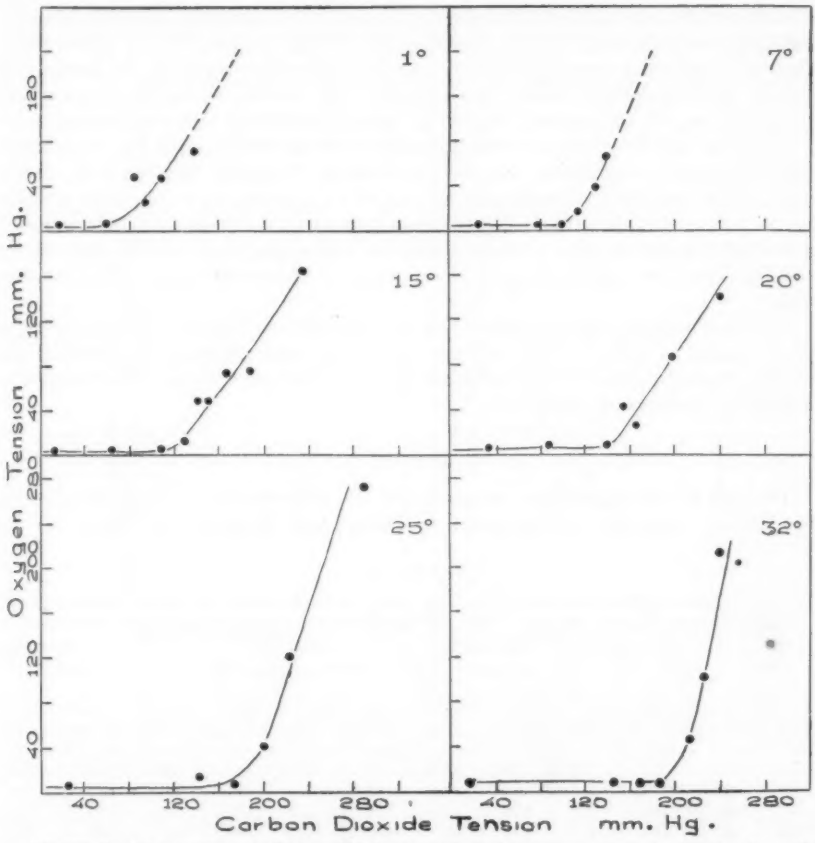

FIGURE 2. The extent to which goldfish can reduce the oxygen content of water in sealed bottles in the presence of dissolved carbon dioxide at various temperatures of acclimation. Each point is the average of three to five determinations.

asphyxiation. As the acclimation temperature is increased the level of carbon dioxide required to limit the level to which the oxygen is used also increases. There is some indication that the rising portion of the curve becomes steeper at the higher acclimation temperatures.

The data from the six curves in Figure 2 are brought together in Figure 3 where the change in sensitivity, i.e., the number of arbitrary units contained within

each curve, is plotted against the temperature of acclimation. The sensitivity of the fish seems to decrease almost directly with the increase in temperature of acclimation.

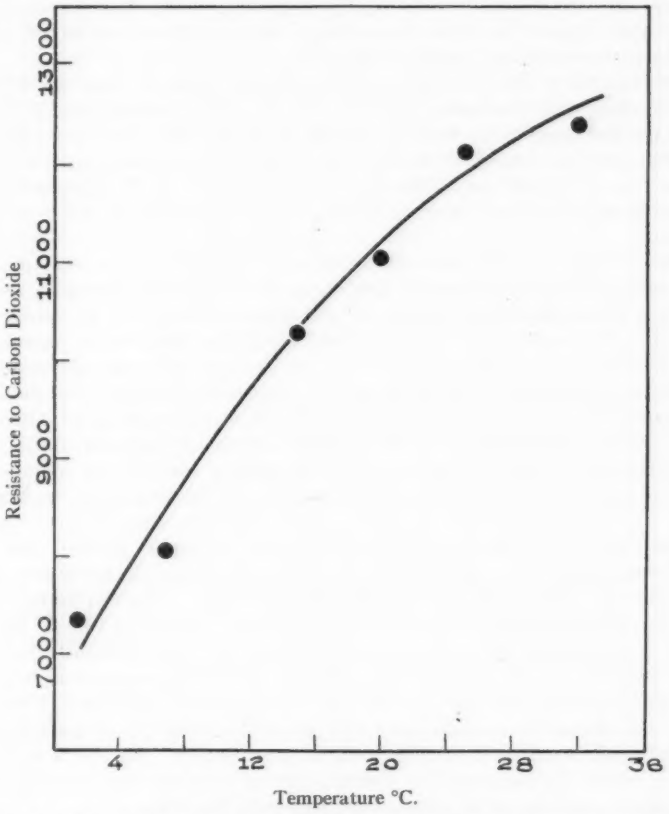

FIGURE 3. The relation between utilization of oxygen in the presence of dissolved carbon dioxide and the temperature to which goldfish are acclimated. The units of resistance to carbon dioxide given are the areas composed by oxygen × carbon dioxide (as mm. Hg) between the resistance curves in Figure 2 and a horizontal line drawn across at 160 mm. Hg oxygen (air saturation). Sensitivity to CO_2 referred to in the text is a reciprocal function of resistance.

DISCUSSION

The ability of the fish to use oxygen in the presence of carbon dioxide as measured here, varies considerably in goldfish adapted to various temperatures within their biokinetic range. The difference between goldfish acclimated to water of 1° C. and those acclimated to water of 32° C. is of the same order as the differences between the common sucker (*Catostomus commersonnii*) and the brown bullhead

(*Ameiurus nebulosus*) when these species are acclimated to about 20° C. (Fry, 1939).

The change in the respiratory sensitivity attendant on change in the environmental temperature is such that when goldfish are adapted to low temperatures their respiratory sensitivity tends toward the type found in cold water species (common sucker): when adapted to high temperatures the respiratory sensitivity resembles that of warm water species (brown bullhead).

Other changes in the respiratory metabolism of fish have been found to be attendant on changes in thermal adaptation (Wells, 1935; Sumner and Wells, 1935; Sumner and Lanham, 1942; Sumner and Doudoroff, 1938). Indeed Sumner and Doudoroff used the change in resistance time to cyanide poisoning as an index of change in thermal adaptation. The change shown in our work appears however to be unique in that the absolute level of respiratory sensitivity is lower at a higher temperature.

While no definite conclusions can be drawn it is interesting to speculate on the change of organization which could bring about this change in respiratory sensitivity. Two phases of the circulatory systems of fish have been shown to be correlated with their respiratory sensitivity. Black (1940) noted that there were significant differences between four species of freshwater fish and the effect of carbon dioxide on the affinity of hemoglobin in whole blood for oxygen, and further, that the fish with the greater sensitivity to carbon dioxide are found in colder habitats. Hart (1945) showed a similar correlation between the stroke output of the heart and the respiratory sensitivity of a series of species. He also pointed out that the influence of the mechanical factor in respiratory transport may at times be so great as to displace the order of respiratory sensitivity of species which might be expected from a comparison of the chemical characteristics of their oxygen transport system. In addition to Hart's material another probable instance of this is found in the marine fish, the tautog (*Tautoga onitis*) and the toadfish (*Opsanus tau*). The respiratory tolerance of these fish is quite different, the toadfish being able to withstand up to 250 mm. of carbon dioxide whereas the tautog can only tolerate 150 mm. (Safford, 1940). The chemical properties of the blood of these fish for transporting oxygen in the presence of carbon dioxide are, however, very similar (Root, Irving, and Black, 1939).

Thus the change in respiratory sensitivity with change in temperature as displayed by goldfish adapted to their thermal environment may be a reflection of changes in either the mechanical or chemical aspects of their circulatory system and the problem remains to see to what extent each may be acting.

There is another change in the organism that may influence the measure of respiratory sensitivity as determined here for the goldfish. The areas enclosed above and to the left of the carbon dioxide-oxygen curves in Figures 1 and 2 are not entirely areas of tolerance in the sense that the animal could remain alive indefinitely under conditions represented by any combination of oxygen and carbon dioxide within these areas. At points in the marginal area near the limiting curve the animal is in a dying condition and its ability to transport oxygen will not depend on its tolerance towards those conditions but upon the length of time its organization can resist the ultimate breakdown. During the part of this period when the ventilatory and circulatory systems are still functioning, sub-minimal amounts of oxygen will be transported to the tissues of the dying animal. While these amounts of oxygen may be sub-minimal from the point of view of maintaining the life of the organism they

may be quite significant in extending the carbon dioxide-oxygen curve considerably beyond the region of tolerance. This effect was well shown by Wiebe *et al.* (1934) who demonstrated that after a fish introduced into a sealed container had extracted oxygen in the presence of a certain pH until it had died, a second subject introduced into the same container could reduce the oxygen still further by a significant amount before it also died.

Thus the decrease in the respiratory sensitivity at higher temperatures as measured by asphyxiation in a sealed bottle could in part be accounted for by increase in resistance of the tissues to oxygen lack or carbon dioxide excess, taking resistance to mean the length of time that an organism can resist a lethal condition before it finally succumbs. However one would not expect this factor to account for anything like the whole of the change in respiratory sensitivity observed.

When conditions are standard with respect to heredity and environment the respiratory sensitivity curves appear to offer a valid physiological yardstick whatever question there may be of exactly what these experiments measure. In the present series of experiments for instance the 20° curve was obtained in 1945 using material of the same age and purchased from the same source as that which was used for the determinations at the other temperatures in 1943, and as will be seen the data are quite consistent.

Necessity for complete acclimation to secure standard results becomes apparent when temperature effect over the biokinetic range at one level of thermal adaptation is considered. We have made two comparisons on fish acclimated to 20° C. In one instance the experiments were conducted in water at 12° C., in the other at 28° C. These data are given in Table II and may be compared with the values given for

TABLE II

The extent to which goldfish acclimated to 20° C. can reduce the oxygen content of water containing dissolved carbon dioxide at 12° C. and at 28° C. Each pair of values is the mean of four determinations

Acclimated to 20° Experiments at 12°		Acclimated to 20° Experiments at 28°	
CO_2 mm. Hg	O_2 mm. Hg	CO_2 mm. Hg	O_2 mm. Hg
24	5.7	28	7.9
82	5.4	69	7.4
100	8.6	121	11
111	9.8	140	18
127	19	157	60
152	32	169	25
181	100	186	141
		217	184

20° C. in Table I. In both cases the respiratory sensitivity proved to be greater than that measured at the temperature of acclimation. The degree of difference that these results show is somewhat surprising in view of the fact that the difference in temperature at which the experiments were carried out was only 8 degrees in either direction from the acclimation temperature, while at the acclimation level chosen the lower lethal temperature is about 18° C. below and the upper lethal 15° C. above the acclimation temperature.

Summary

1. By asphyxiating goldfish in sealed bottles of water containing dissolved carbon dioxide and oxygen, it was found that the ability of the fish to use oxygen in the presence of carbon dioxide increases as the acclimation temperature of the fish is raised.

2. Goldfish acclimated at 1° C. are limited in their ability to use oxygen when a tension of 60 mm. or more carbon dioxide is present in the water, whereas goldfish acclimated at 32° C. are not limited in the utilization of oxygen unless the carbon dioxide tension is 200 mm. or more. Fish at intermediate temperatures of 7°, 15° 20° and 25° C. begin to show a decrease in the ability to use oxygen when they are in the presence of carbon dioxide tensions of 100, 120, 140, and 170 mm. respectively.

3. Reliable results could be obtained only after complete acclimation of the fish to the temperature at which the experiments were made. However, with acclimated fish of similar stock the result could be reproduced from year to year at the same season.

4. The explanation for this change in sensitivity to carbon dioxide in acclimated fish lies possibly in the effects of temperature on both the mechanical transport of the blood and the chemical transport of oxygen by the blood.

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